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Dynamiques individuelles et interactions entre santé mammaire, déséquilibres nutritionnels et l'établissement de la gestation chez la vache laitière

RESUME

La fertilité des vaches laitières s'est beaucoup dégradée au cours des dernières décennies, tant à l'échelle nationale qu'internationale. L'objectif poursuivi est d'apporter un éclairage épidémiologique sur les relations dynamiques entre la conception (réussite à l'insémination artificielle ; IA), les mammites subcliniques et les déséquilibres métabolico-nutritionnels que sont la cétose subclinique et les déséquilibres azotés de la ration.

Un premier chapitre bibliographique dresse l'état de l'art des liens entre performances de reproduction et (i) mammites, (ii) cétose subclinique et (iii) excès de protéines dégradables, en identifiant clairement les interactions et associations croisées entre ces différents composants, et en quantifiant autant que possible ces liens.

Les trois parties suivantes mobilisent des données exhaustives du contrôle laitier français sur la période 2008-2012, qui ont été confrontées aux données d'IA. La variable d'intérêt retenue est la conception après IA, soit pour la première IA (IA1) ou pour toutes les IA. Les variations des concentrations de cellules somatiques (CCS) autour de l'IA ont été utilisées pour décrire la dynamique des mammites subcliniques autour de l'IA, selon 4 classes (Bas-Bas, Bas-Haut, Haut-Bas et Haut-Haut) et pour différents seuils de CCS. Le statut de cétose subclinique a été évalué grâce aux taux butyreux et protéique du lait. Les concentrations d'urée du lait sont utilisées pour caractériser les déséquilibres azotés.

Le second chapitre propose une description succincte des résultats de production et de reproduction des troupeaux bovins laitiers français sur la période d'étude.

Le troisième chapitre focalise sur l'interaction entre les CCS et la cétose subclinique dans un modèle de régression de Poisson expliquant la conception. Les chances de conception à l'IA1 sont réduites de 14% (Risque relatif = 0,86 [IC 95%=0,85–0,87]) pour les groupes Bas-Haut et Haut-Haut, comparé au groupe Bas-Bas, et de 3 à 17 %, selon les définitions retenues, lors de cétose subclinique comparé à son absence. Les résultats identifient et quantifient clairement l'interaction entre la cétose subclinique et les mammites subcliniques dans leur association avec la conception : la baisse de la conception est jusqu'à 2 fois supérieure lors de la présence simultanée d'une augmentation des CCS et d'une cétose subclinique par rapport à la situation où il y a seulement augmentation des CCS.

Le quatrième chapitre, mobilisant des méthodes proches de celles du chapitre précédent, montre que la baisse de la concentration d'urée dans le lait autour de l'IA (en dessous de 150 mg/kg, 2,6 mmol/L) est associée à une baisse de la conception de 5 à 9% (Risque relatif = 0.91 (IC95%=0,87-0,96)]) par rapport à des concentrations d'urée du lait qui restent stables (250 - 450 mg/kg ou 4,3-7,7 mmol/L). Ceci révèle l'importance de la stabilité du métabolisme azoté autour de la conception, y compris pour des variations d'urée du lait ou du sang à la baisse, alors que seule la hausse de la concentration d'urée était identifiée comme un facteur de risque de dégradation des résultats de reproduction jusqu'alors.

La dernière partie permet de mettre en perspective ces éléments originaux. Une des principales limites de ces travaux est la définition imprécise de la cétose subclinique réalisée à partir des taux du lait ; les résultats actuels bénéficieraient d'une actualisation avec un indicateur plus précis de ce trouble. Ces résultats soutiennent qu'une inflammation locale peut affecter la réponse de l'ensemble de l'organisme et altérer les fonctions d'autres organes dans les semaines qui suivent son apparition. Ils illustrent la complexité et les interactions entre les différents troubles chez un même animal. Par ailleurs, maintenir des concentrations basses de l'urée est légèrement pénalisant pour la conception, et ne garantit pas de meilleurs résultats de reproduction.

Mot clés : bovins laitiers, fertilité, mammite, cétose subclinique, urée

Individual dynamics and interactions of udder health, nutritional disorders and conception in dairy cows

ABSTRACT

Reproductive performances of dairy cows are recognized as a key parameter for the profitability of dairy farms, but they are getting worse continuously in many countries during the last decades. Infectious and nutritional disorders are possible contributors to these changes. Mammary infection, nitrogen imbalance and metabolic disorders have been reported to be negatively associated with conception, but their interactions and dynamics are not fully understood. The objective of the present work is to better describe the relationship between fertility, udder health, subclinical ketosis (SCK) and nitrogen imbalance accounting for the temporal variations of these events and their interactions.

The first section reviews the link between reproductive performances and (i) mastitis, (ii) metabolic disorders and (iii) nitrogen imbalance in dairy cows in order to clarify the complex interaction among these events.

The three other sections are based on exhaustive data from the national French dairy milk improvement system and data on the artificial inseminations (AI) from 2008 to 2012. Fertility was explained as conception at the first (AI1) or at all AI. The udder health status was evaluated through the somatic cells counts (SCC). Several proxies based on the milk fat and protein contents were proposed to define SCK. Milk urea concentration was used to investigate the exposure to nitrogen imbalance.

The second section aims to describe the actual situation of milk production and reproduction in French dairy herds.

The third section focuses on the interaction between SCC and SCK and their association with conception. On average, the risk of conception at AI1 was 14% lower when the SCC increased or remained high within 40 days before and after AI (Relative risk [and 95% CI] = 0.86 [0.85–0.87]), compared to low SCC before and after AI. The reduction in conception rate associated with SCK (fat and protein contents changes) varied from 3% to 17% depending on the SCK proxy used. Including the interaction term SCC*SCK clearly showed that the association of increased SCC around AI with conception was modified by the presence of SCK. A cow that already has SCK and experiences an increase in SCC around or after AI exhibits up to 2 times further decrease in conception, compared with a cow with a high SCC and no SCK.

The fourth section, using similar methods as the previous one, shows that low milk urea concentrations after AI are negatively associated with conception. Cows with a low urea (< 150 mg/kg, 2.6 m*M*) after AI have a reduced conception (Relative risk RR [and 95% CI] = 0.96 [0.94–0.99]) compared to cows with intermediate urea (250-450 mg/kg, 4.3 - 7.7 m*M*) after AI. Furthermore, the risk of conception was 5 to 9% lower (relative risk [and 95% CI] = 0.91 [0.87–0.96]) when urea concentrations decrease from intermediate before to low after AI, compared with cows with constant intermediate urea values. This work revealed that a decrease in urea from intermediate (before AI) to low (after AI) is a risk factor for conception failure in addition to the previously known risk factor that is nitrogen excess.

The final section aims to highlight the perspectives of these results. The definition of SCK used in this work was identified as the main limitation and the present results would benefit from an update with a better indicator of this disorder. The present work supports that a local inflammation may affect the entire body response and alters the functions of other organs like those of the reproductive tract. Furthermore, maintaining low urea concentrations does not provide any particular advantage and might be negatively associated with conception.

Key words: dairy cows, reproduction, mastitis, ketosis, urea

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LISTE DES COMMUNICATIONS

Articles à comité de lecture :

- **Albaaj A.**, Foucras G., Raboisson D. 2017. High somatic cells counts and changes in milk fat and protein contents around insemination are negatively associated with conception in dairy cows. Theriogenology, 88: 18-27. doi: 10.1016/j.theriogenology.2016.09.043.
- **Albaaj A.**, Foucras G., Raboisson D. 2017. Changes in milk urea around insemination are negatively associated with conception success in dairy cows. J. Dairy Sci., 100: 3257-3265. doi: 10.3168/jds.2016-12080.
- Raboisson D., **Albaaj A.**, Nonne G., Foucras G. 2017. A meta-analysis on high urea and pregnancy or conception in dairy cows. J Dairy Sci. 2017 Jun 28. pii: S0022-0302(17)30615-X. doi: 10.3168/jds.2016-12009.

Communications orales :

- **Albaaj A.**, Foucras G., Raboisson D. Occurrence of mastitis around insemination reduces establishment of pregnancy. European Buiatrics Forum (EBF), 14-16 October 2015, Rome, Italia.
- **Albaaj A.**, Foucras G., Raboisson D. Interaction between conception, mastitis and subclinical ketosis in dairy cows. International Congress of Production Diseases in Farm Animals (ICPD), 20-23 June 2016, Wageningen, Netherlands.

Posters :

- **Albaaj A.**, Foucras G., Raboisson D. Mammites, cétose subclinique et fertilité chez les bovins : apports épidémiologiques. Colloque JAS, 9-12 mai 2016, Poitiers, France.
- **Albaaj A.**, Raboisson D., Foucras G. Conception is all the more reduced that mastitis and subclinical ketosis simultaneously occur around artificial insemination. The 29th World Buiatrics Congress (WBC), 3-8 July 2016, Dublin, Ireland.

Publications dans des journées de recherche :

- Raboisson D., **Albaaj A.**, Foucras G. L'association entre mammites et fertilité est modulée par la présence de cétose subclinique. Journées nationales GTV, 2016, Nantes, France.

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LISTE DES ABRÉVIATIONS

AGNE : *Acides gras non estérifiés*
AI : *Artificial insemination*
AI1 : *First artificial insemination*
BCS : *Body condition score*
BHBA : *Acide bêta-hydroxybutyrique / beta-Hydroxybutyric acid*
BUN : *Blood urea nitrogen*
CCS : *Concentrations de cellules somatiques*
CK : *Clinical ketosis*
CM : *Clinical mastitis*
cNEB : *Calculated negative energy balance*
CP : *Crude protein*
CR : *Conception rate*
DIM : *Days in milk*
DM : *Dry matter*
DPA : *Dairy production area*
EB : *Energy balance*
FPR : *Fat : protein ratio*
FSH : *Hormone folliculo-stimulante / Follicle-stimulating hormone*
GH : *Hormone de croissance / Growth hormone*
HR : *Hazard ratio*
IA : *Insémination artificielle*
IA1 : *Insémination première*
IAF : *Insémination fécondante*
IGF-1 : *Insulin-like growth factor-1*
LH : *Hormone lutéinisante / Luteinizing hormone*
MCP : *Milk Control Program*
MIR : *Spectrométrie moyen infrarouge*
MJ : *Megajoule*
m*M* : *mmol/L*
MUN : *Milk urea nitrogen*
MY : *Milk yield*
NEB : *Negative energy balance*
NEFA : *Non-esterified fatty acids*
OR : *Odds ratio*
PGF2α : *Prostaglandine F2α / Prostaglandin F2alpha*
PMN : *Polymorph-nuclear cells*
PUN : *Plasma urea nitrogen*
RDP : *Rumen degradable protein*
RR : *Relative risk*
RUP : *Rumen undegradable protein*
SCC : *Somatic cells count*
SCK : *Subclinical ketosis*
SCM : *Subclinical mastitis*
SCS : *Somatic cells score*
TB : *Taux butyreux*
TP : *Taux protéique*
VWP : *Voluntary waiting period*

INTRODUCTION GÉNÉRALE

La production laitière et la reproduction sont deux fonctions clés de la rentabilité de l'élevage bovin laitier (Groenendaal et al., 2004; Meadows et al., 2008). Elles sont fortement sollicitées dans les systèmes de production intensifs, tout en restant déterminantes dans l'équilibre technique et économique des productions. Le contexte économique de la production laitière a été profondément modifié durant les dernières décennies. L'ouverture internationale du marché conduit par exemple à une volatilité des prix du lait, questionnant en particulier les coûts de production. Les élevages laitiers ont connu une intensification majeure au cours des dernières décennies, associée à une sélection génétique poussée, des progrès dans l'alimentation des animaux et une amélioration de la gestion zootechnique des troupeaux. La France, bien qu'en faible régression de collecte, reste aujourd'hui le 2ème producteur européen de lait après l'Allemagne avec 25,3 mds de litres de lait et le lait de vache représente 97% de ce volume (http://agreste.agriculture.gouv.fr/).

Les paramètres de reproduction des bovins laitiers se sont détériorés sur cette même période. La fertilité et la fécondité des vaches laitières se dégrade continuellement, tant à l'échelle nationale qu'internationale. Cette dégradation des résultats de reproduction dans les troupeaux laitiers est évoquée dans différentes enquêtes en France, et elle a été plus forte et plus rapide dans la race Prim'holstein que dans les races Normande et Montbéliarde. La dégradation de la fertilité représente un enjeu majeur des élevages bovins laitiers car elle conduit à des besoins supplémentaires en animaux de renouvellement (Boichard, 1988), eux-mêmes coûteux pour remplacer les vaches non-productives et réformées précocement (Mohd Nor et al., 2015).

De très nombreuses études ont contribué à comprendre les facteurs de risque de la dégradation des performances de reproduction, ainsi que les mécanismes associés, et à proposer divers outils pour les améliorer. Favoriser la reprise de la cyclicité, améliorer la détection des chaleurs, inséminer au bon moment, limiter les risques de non fécondation et de mortalité embryonnaire représentent les principaux leviers pour améliorer la reproduction et la fertilité. Ces différents leviers représentent une mobilisation de moyens qui doivent être justifiés par le retour financier permis via l'amélioration des résultats. La gestion économiquement raisonnable de la reproduction reste complexe en raison des multiples interactions entre production et maladies, ces dernières influençant la fonction de reproduction.

De nombreux facteurs sont associés à la dégradation des paramètres de reproduction actuellement observée : sélection génétique, production laitière, changement des conditions d'élevage, conduite alimentaire, … Cependant, les mécanismes d'action qui les relient ne sont pas tous clairement identifiés.

Une corrélation génétique négative entre le niveau de production laitière et les fonctions de reproduction a été décrite (Hansen, 2000). Les corrélations génétiques entre le taux de réussite à l'insémination artificielle (IA) et la production laitière chez les trois principales races laitières françaises ont par exemple été estimées à environ -0,11 à -0,32 (Boichard and Barbat, 1998). L'accroissement de la production laitière est associé à un risque fort d'une cyclicité anormale (Disenhaus et al., 2002), à une baisse du taux de réussite de la première IA (IA1) (Espinasse et al., 1998), à une probabilité élevée de retour en chaleurs après l'IA1 (Hery et al., 1995), à l'augmentation des intervalles entre le vêlage et l'IA1 (Hillers et al., 1984; Coleman et al., 1985) et à la mortalité embryonnaire tardive (Michel et al., 2003).

La nutrition constitue une des composantes déterminantes de la santé du troupeau et joue un rôle primordial dans la maîtrise de la reproduction. La couverture des besoins des vaches hautes productrices s'avère impossible en début de lactation et la vache présente un bilan énergétique négatif dont la force et la durée varient d'une vache à l'autre en fonction des apports alimentaires, du niveau de production laitière et de l'état des réserves corporelles disponibles au vêlage (Chilliard, 1987; Villa-Godoy et al., 1988). Ce déficit dure généralement de 5 à 10 semaines (Jorritsma et al., 2003). La cétose est un trouble du métabolisme énergétique qui correspond à l'accumulation des corps cétoniques et de nutriments énergétiques habituels dans le sang des ruminants. Cette accumulation est observée lors de déficit énergétique trop intense. Le déficit énergétique et la cétose subclinique sont connus comme des facteurs qui peuvent détériorer les fonctions de reproduction. Le risque de cétose subclinique est entre autres lié à l'adéquation entre le niveau de production et les apports alimentaires, créant ainsi des liens complexes entre reproduction, alimentation et production laitière.

Le métabolisme énergétique interagit par ailleurs avec le métabolisme azoté, et l'équilibre de la ration en protéines peut affecter considérablement les performances de reproduction. La grande majorité des travaux a étudié l'impact du déficit azoté sur les performances de reproduction car la supplémentation protéique, entre autres en début de lactation, est une pratique largement adoptée pour augmenter la production laitière dans les élevages laitiers. Cet excès a été identifié comme néfaste sur les performances de reproduction (Canfield et al., 1990; Edwards et al., 1980; Barton et al., 1996), de manière directe ou indirecte via l'exacerbation du déficit énergétique.

Le peripartum est une période sensible pour de multiples maladies à composante infectieuse. Le risque élevé d'occurrence de ces maladies dans cette période provient notamment de la baisse d'immunité, lié au stade physiologique et aux profils hormonaux, mais aussi à la présence fréquente d'une cétose subclinique. Cette immuno-dépression transitoire favorise divers troubles (métrites, boiteries, mammites…), qui sont à leur tour des facteurs de risque de dégradation des résultats de la reproduction. Dans les 2 semaines post-partum, 20-40% des vaches développent une métrite chronique, 20% une endométrite et 20-30% une mammite (Sheldon et al., 2009; Zhao and Lacasse, 2008).

Les infections mammaires ont été clairement identifiées comme un facteur de risque de la baisse de la fertilité. Les mammites constituent le trouble sanitaire le plus fréquent des vaches laitières et sont souvent considérées comme une des causes de pertes économiques les plus importantes parmi les troubles du peripartum (McInerney et al., 1992). La diminution moyenne de la production de lait dues aux mammites cliniques a été évaluée à 700 kg par vache et par an (Seegers et al., 2003). Pour chaque multiplication par 2 de la concentration des cellules somatiques (CCS) au-delà de 50 000 cellules/mL de lait, les baisses de production laitière ont été établies à 80 kg (1,3%) et à 120 kg (1,7%) chez les primipares et les multipares respectivement (Hortet and Seegers, 1998a). L'apparition d'une mammite clinique ou subclinique a été identifiée comme un facteur de risque de non réussite à l'IA (Loeffler et al., 1999a) et de l'allongement des intervalles vêlage-IA1 et vêlage-insémination fécondante (IAF)(Santos et al., 2004).

Dans ce contexte, il apparaît important de reconsidérer l'ensemble des facteurs de risque influençant la fonction de reproduction de manière plus globale et d'intégrer les différentes interactions entre les facteurs de risque de troubles de la reproduction ou de dégradation des performances de reproduction. L'évolution du contexte technique et économique conduit à s'interroger d'une part sur la fréquence de ces facteurs de risque, et d'autre part sur les possibilités d'adaptation des animaux et des éleveurs à ces troubles et à la baisse des performances. La validation épidémiologique des liens entre ces entités zootechniques et sanitaires est importante, car elle permet de hiérarchiser les facteurs de risque des troubles de reproduction en élevage, de définir les outils diagnostiques de ces maladies métaboliques et d'identifier les incidences et prévalences acceptables au plan économique. En effet, comprendre le poids relatif des différents facteurs de risque, au sein du complexe métabolico-infectieux du peripartum, dans les performances de reproduction s'avère une étape importante pour un conseil en élevage efficace et dans le contexte plus global de la médecine de précision. De plus, la quantification de ces relations représente un enjeu-clé pour la calibration de futurs modèles épidémiologiques ou bioéconomiques. Dans les deux cas, ces avancées devraient permettre de faciliter la prise de décision et d'en améliorer la pertinence.

Les performances de reproduction à l'échelle de la vache ou du troupeau s'apprécient par l'évaluation de deux critères, la fertilité et la fécondité. La fertilité peut se définir comme la capacité à se reproduire, ce qui correspond chez la femelle à la capacité à produire des ovocytes fécondables. La fécondité caractérise l'aptitude pour une femelle à mener à terme une gestation dans les délais requis. La fécondité englobe donc la fertilité et comprend le développement embryonnaire et fœtal, la mise bas et la survie du nouveau-né. Il s'agit d'une notion avec une composante économique, ajoutant à la fertilité un paramètre de durée (Chevallier and Champion, 1996).

Les paramètres de fertilité les plus couramment utilisés sont :

- Le nombre d'IA(s) par IAF : cet indice de fertilité s'obtient en divisant, au niveau de la population, le nombre total d'IA par le nombre d'IA fécondantes. Le nombre de vêlages est parfois utilisé au lieu du nombre d'IAF, mais il est alors biaisé par les évènements ultérieurs à la fécondation (avortements …). Le nombre total d'IA est la somme des IA réalisées sur les vaches fécondées, non fécondées ou réformées sans que l'on puisse déterminer avec certitude si elles sont gravides ou non. Ce critère reflète le nombre d'IA nécessaires au niveau d'une population pour obtenir un vêlage.
- Le taux de réussite en IA1 : il peut être mesuré *a posteriori* par le pourcentage de non-retour en chaleurs à 60 et 90 jours.
- Le taux de vêlages à l'IA1 : ce critère se calcule en divisant le nombre de vêlages obtenus dans une durée compatible avec la durée de gestation après l'IA1 par le nombre d'IA1. Il donne une bonne idée de la fertilité globale du troupeau mais il est biaisé par le nombre de vaches sorties de l'exploitation ou de l'échantillon après une seule IA (morte, vendue, réformée) sans évaluation de leur état de gestation.
- Le pourcentage de vaches inséminées plus de 2 fois : il peut être un marqueur plus précis du type de gestion des réformes car il dépend de la politique de réforme des troupeaux.
- Le taux de vêlages global : ce critère est peu utilisé et il se calcule en divisant le nombre de vêlages obtenus au cours de la campagne de reproduction par le nombre de femelles mises à la reproduction. Il est biaisé par le nombre de vaches sorties de l'exploitation ou de l'échantillon (morte, vendue, réformée) sans évaluation de leur état de gestation.

Les principaux paramètres dérivés d'intervalles décrivent la fécondité. On retiendra essentiellement :

- L'intervalle vêlage-première insémination (IV-IA1) : cet intervalle traduit le délai de mise à la reproduction, il reflète à la fois la reprise de la cyclicité mais aussi la qualité de la détection des chaleurs et la politique de l'éleveur (IA précoces ou tardives).
- L'intervalle vêlage-insémination fécondante (IV-IAF) : (Days open ou interval between calving to conception en anglais). Les vaches non fécondées en IA1 reviendront en chaleurs de façon régulière ou irrégulière. La majorité d'entre elles doit avoir un retour en chaleurs régulier (compris entre 18 et 24 jours). Les retours entre 36 et 48 jours sont probablement réguliers, mais signent un défaut de détection. Cet intervalle peut être assimilé à la période de reproduction. Il ne peut être calculé que sur les vaches dont la gestation a été confirmée.
- L'intervalle vêlage-vêlage (IV-V) : c'est le critère technico-économique le plus intéressant en production laitière. Il représente le temps nécessaire pour féconder une vache et combine le temps de mise à la reproduction, le temps perdu en raison des échecs à l'IA et la durée de la gestation. Il exclut par défaut les vaches réformées avant le vêlage suivant (y compris si gestantes).

L'objectif poursuivi dans ce travail est de préciser les associations directes et croisées entre les performances de la reproduction, et les infections mammaires ou les déséquilibres alimentaires de la vache laitière, en focalisant sur la dynamique de ces relations et sur les conditions environnementales les favorisant ou les limitant, à partir de bases de données exhaustives françaises. En raison de la nature des données disponibles, le principal indicateur retenu est la conception des vaches à l'IA, défini comme la réussite à l'IA.

La première étape de ce travail a consisté en une étude bibliographique qui détaille les principaux facteurs de risque responsables de la dégradation des paramètres de fertilité chez la vache laitière et les mécanismes explicatifs sous-jacents. Un objectif revendiqué de cette revue de littérature est la quantification des relations étudiées et la mise en avant des conditions de validité de ces relations. La problématique de la partie expérimentale sera alors présentée à la fin de cette synthèse bibliographique.

La deuxième partie présentera les résultats des performances productives et reproductives du cheptel bovin laitier français, et précisera quelques éléments de qualité du lait. Le 3ème chapitre présentera, sous la forme d'un article scientifique, l'association entre les infections mammaires et la fertilité, analysée via la dynamique des CCS autour de l'IA en présence ou non de la cétose subclinique. Le

4ème chapitre sera consacré au déficit azoté tel qu'il peut être défini par la mesure des valeurs de concentration d'urée dans le lait. Sous la forme d'un article scientifique, l'association négative entre fertilité et concentration en urée basse bien que modérée, sera explicitée. Enfin, l'ensemble sera mis en perspectives et discuté dans une dernière partie.

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1. Chapitre 1. Comment la reproduction, les mammites et les déséquilibres alimentaires interagissent entre eux ? Une revue de la littérature

Résumé :

L'objectif poursuivi ici est de synthétiser les principaux travaux sur les facteurs de risque de la dégradation de la fertilité chez la vache laitière, et d'en préciser les mécanismes explicatifs sous-jacents, en quantifiant, lorsque cela est possible, la force de ces relations.

La baisse régulière de la fertilité observée dans le monde entier est très souvent associée et attribuée à l'augmentation de la production laitière observée en parallèle, mais des facteurs de confusion sont identifiés. La survenue d'une mammite clinique après l'IA et la présence d'une mammite subclinique autour de l'IA réduit les chances de conception. L'amplitude de cette diminution de la conception dépend de plusieurs paramètres dont la sévérité de l'inflammation.

Le déficit énergétique et l'hypercétonémie sont reconnus de manière consensuelle comme un facteur de risque de dégradation des performances de reproduction, mais la quantification de la relation reste imprécise. En cas d'hypercétonémie, par comparaison avec son absence, le risque relatif [IC95%] de réussite à l'IA est de 0,62 [0,41-0,93] à 0,67 [0,53-0,83], celui de métrite puerpérale est de 1,91 [1,72-2,12], celui de rétention placentaire de 1,51 [1,19-1,92] et le rapport des risque lié à la conception est de 0,77 [0,61-0,97].

L'excès de protéines dégradables de la ration, associée à une augmentation de la concentration d'urée dans le lait ou dans le sang, est considéré comme affectant négativement la fertilité. Les chances de conception sont 43% moins élevées lorsque l'urée $\geq$ 7,0 mmol/L dans le sang ou $\geq$ 420 mg/kg dans le lait. Le seuil de 6,5 mmol/L ne peut être formellement exclu. L'exposition avant l'IA semble être plus néfaste sur la conception, bien que ce point ne soit pas totalement éclairé. Peu de données lient un déficit en azote ou une baisse de l'urée à une dégradation des performances de reproduction.

En préparation pour publication

How reproduction, mastitis and some nutritional disorders interact: an overview

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1.1. Introduction

The dairy sector around the world is undergoing major changes. The main emphasis for dairy farmers is to sell as much milk as possible with maximum efficiency, which resulted in more intensive management systems. The ongoing changes in the dairy systems result in new major challenges for farmers in terms of animal welfare. High yields are shown to be associated with several health troubles that might affect the dairy cow fertility, with mastitis and negative energy balance (NEB) being especially deleterious. Most infectious diseases and metabolic disorders occur during the transition and early lactation periods. Thus, dairy cow management and nutrition within these periods have received particular interest in recent years. The purpose of this section is to address in details the complex relationship between the health status and the reproductive performance of dairy cows in the early lactation and to reveal the possible mechanisms that link these factors together.

1.2. Mastitis and reproduction in dairy cows

1.2.1. Definition and risk factors

Intramammary infections caused by environmental pathogens are very common in early lactation cows and several studies have demonstrated that high-producing cows are at an increased risk of infectious diseases (Gröhn et al., 1989; Oltenacu et al., 1991; Oltenacu and Ekesbo, 1994). However, the incidence and prevalence of these infections may vary amongst countries and could be affected by factors such as age, stage of lactation, season, and herd size. Mastitis is a multifactorial inflammation of the mammary gland that is induced by the entrance of a pathogenic microorganism into the udder through the teat canal. Its incidence depends on several risk factors such as the exposure to a source of contamination and microbes, the udder defense mechanisms and presence of environmental risk factors

(Suriyasathaporn et al., 2000). It has been considered as the most imposing disorder in this period since it affects an important proportion of high yielding dairy cows worldwide.

It may present as either clinical or subclinical infections. The clinical form has visible symptoms and may be characterized by abnormal milk secretions and the severe cases are associated with local or systemic signs of inflammation such as heat, fever and loss of appetite, and its prevalence is estimated to range between 8.1 to 13% (van den Borne et al., 2007; Tenhagen et al., 2009; Barkema et al., 1999).

The subclinical form is characterized by the absence of visual signs of infection and it is the most prevalent form of mastitis where its prevalence has been estimated at 5 - 30% or even higher (Contreras et al., 2007). Subclinical mastitis (SCM) could be diagnosed by measuring some parameters such as the electric conductivity and lactose content of the milk. It also can be diagnosed by the detection of pathogens in bacteriological cultures of aseptically-collected milk samples, but these methods are not practically feasible as routine tests. However, the often used method is to measure the somatic cell count (SCC) or a cellular logarithmic scale of it called the somatic cell score (SCS) in the milk as an indirect indicator of the mammary health (Shook and Schutz, 1994; Mrode and Swanson, 2003). A meta-analysis conducted to define the SCC from a healthy udder defined this level to be about 70,000 cells/mL (Djabri et al., 2002) depending on parity, breed, days in milk (DIM) and the level of milk production. In addition, a threshold of 200,000 cells/mL has been proposed to distinguish SCM from healthy udders (Dohoo and Leslie, 1991), although other thresholds are used in some studies depending on the previously mentioned factors (Deluyker et al., 2005; Halasa et al., 2009; Archer et al., 2013). Mastitis can also be classified according to the duration of infection and the pathogen-dependent immune responses into acute or chronic forms. A sudden onset defines acute cases and chronic mastitis is characterized by an inflammatory process that lasts for months and results in progressive development of fibrous tissues (Aitken et al., 2011).

A large number of bacterial pathogens may cause mastitis in dairy cows. This variety may give rise to longer or shorter periods of subclinical infections before the outbreaks of clinical mastitis (CM) (Hagnestam et al., 2007). Among primiparous cows, *Staphylococcus aureus, Escherichia coli, Klebsiella spp.,* caused the greatest losses in milk yield (MY). Among older cows, *Streptococcus spp., Staph. aureus, Trueperella pyogenes, E. coli, and Klebsiella spp.* caused the most significant losses (Gröhn et al., 2004).

Milk yield reduction associated with CM has been found to be greater in lactating cows than in heifers (Bartlett et al., 1991; Hortet and Seegers, 1998b; Gröhn et al., 2004). In multiparous cows, the risk of developing CM increases with increasing parity (Steeneveld et al., 2008). The incidence of mastitis reported to be 14.0, 20.9 and 25.9% for parities 1, 2 and 3, respectively (Pritchard et al., 2013) and cows of later parities developed several episodes per lactation. Within the same parity, higher producing cows were more likely to be diseased (Rajala-Schultz et al., 1999; Gröhn et al., 2004; Wilson et al., 2004). Higher prevalence of CM was observed in early lactation cows and the infection was more severe than in cows diseased in mid or late lactation (Hortet and Seegers, 1998b), whereas the risk of SCM increased with increasing DIM post-partum (Busato et al., 2000). Previous occurrences of mastitis increase the risk of a cow developing a new case of mastitis in the subsequent lactations (Steeneveld et al., 2008). Other peripartum disorders, such as dystocia, milk fever, retained placenta, metritis, ketosis, and lameness, are also reported to increase the risk of mastitis (Emanuelson et al., 1993; Svensson et al., 2006).

1.2.2. Mastitis and milk production

A clear evidence about a positive correlation between mastitis (both clinical and subclinical) and MY has been established and numerous studies found unfavorable genetic correlations between MY and CM (Bunch et al., 1984; Emanuelson et al., 1988), suggesting that the improvement of milk production consequently increased the incidence of CM. However, the magnitude of the infection and the extent of MY loss depend on several factors including the causative pathogen, cow parity, the stage of lactation, and the severity and the duration of the disease …etc. This may contribute to the wide range of losses reported in the literature and synthetized (Table 1) in a previous review (Seegers et al., 2003). In brief, mean lactational losses in MY ranged from 31 to 749 kg depending on the parity and the time of occurrence. Lactational 305-days losses of milk, fat, and protein yields were estimated to reach up to 9, 8 and 7% in primiparous cows and 11, 12 and 12% in multiparous cows, respectively, when developing a CM (Hagnestam et al., 2007). The effect of CM on fat and protein yield seemed to be caused by reduced milk production and not by changes in the fat and protein content of the milk.

Table 1. Summary of study samples and the results in studies dealing with milk loss due to clinical mastitis at lactation level and published since 1990; source: (Seegers et al., 2003)

Reference	Animals			Loss		Comment
	Parity	Milk (kg/lact)	Breed[1]	Mean (kg)	%	
(Houben et al., 1993)	1	6,433	H	31 to 128	0.5 to 2.0	Effect of 1 to > 3 quarter-cases with incurrent lactation
(Myllys and Rautala, 1995)	1	5,564	A & F	32.8	0.6	Mastitis only from 7 d. before to 7 d. after calving
(Pedraza, 1991)	1	4,639	H	749	–[2]	
(Rajala-Schultz and Grohn, 1999)	1		A	294, 348, 110	–[2]	Effect of occurrence before peak, between peak and 120 d. and later, respectively
(Houben et al., 1993)	2	7,632	H	155 to 448	2.0 to 5.8	Effect of 1 to > 3 quarter-cases with incurrent lactation
(Wolf and Jahnke, 1990)	2	4,572	H	205	4.4	
(Rajala-Schultz and Grohn, 1999)	2		A	284, 300, 220	–[2]	Effect of occurrence before peak, between peak and 120 d. and later, respectively
(Firat, 1993)	> 2	6,027	H	231	3.8	
(Pedraza, 1991)	> 2	5,256	H	734	–[2]	
(Houben et al., 1993)	3	8,286	H	NS[3]	NS	Effect of 1 to > 3quarter-cases with incurrent lactation
(Rajala-Schultz and Grohn, 1999)	3		A	509, 352, 387	–[2]	Effect of occurrence before peak, between peak and 120 d. and later, respectively
(Rajala-Schultz and Grohn, 1999)	> 3		A	552, 329, 357	–[2]	Effect of occurrence before peak, between peak and 120 d. and later, respectively
(Hoblet et al., 1991)	all	8,430	H & J	75-206	0.9–2.4	
(Lescourret and Coulon, 1994)	all	5,032	H & M	313 ± 207	6.2	Summarized by reviewers

[1] H: Holstein-Friesian or Friesian; A: Ayrshire; J: Jersey; M: Montbéliarde.
[2] Calculation not applicable due to no data on incidence rate or lactational yield presented in the paper.
[3] Not significant.

The reduction in MY associated with SCM has been estimated in a review to reach 80 kg (1.3%) and 120 kg (1.7%) per 2-fold increase in SCC above 50,000 cells/mL in primiparous and multiparous cows, respectively (Hortet and Seegers, 1998a). Figure 1 displays a summary of the reduction in MY at the lactation level associated with SCC levels in primiparous and multiparous cows.

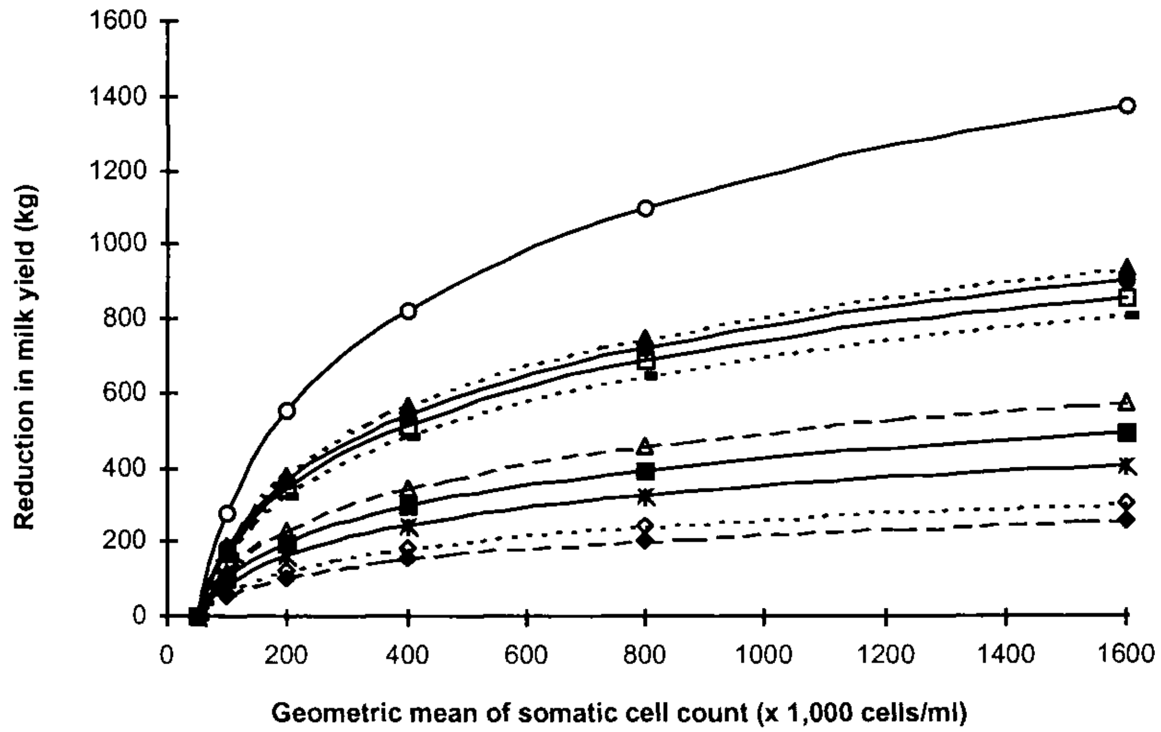

Figure 1. Loss in milk yield at the lactation level expressed in deviation from yield of cows with a geometric mean of SCC lower than 50,000 cell/mL; source: (Hortet and Seegers, 1998a)
….. multiparous cows; – – – – primiparous cows; —— multiparous and primiparous cows; ▲: (Raubertas and Shook, 1982); Δ: (Raubertas and Shook, 1982); ●: (Dentine and McDaniel, 1983); ■: (Dohoo and Martin, 1984); □: (Salsberg et al., 1984); ○: (Serieys, 1985); ◊: (Batra, 1986); ♦: (Batra, 1986); *: (Youl and Nicholls, 1988); -: (Fetrow et al., 1991)

These results were confirmed by more recent studies evaluating the udder inflammation through different levels of SCC as summarized in Table 2. Losses in daily milk yield increased when SCC increased above the threshold of 50,000 or 100,000 cell/mL, and these losses were more severe in multiparous than in primiparous cows.

Table 2. A summary of reported daily milk loss (kg) for specific SCC ($\times 10^3$ cells/mL) values in previously published studies

Reference	Breed	Parity	SCC ($\times 10^3$)	Loss in daily MY (kg)
(Hortet et al., 1999)[1]	Holstein	1	>200	0.31
			>600	0.79
(Dürr et al., 2008)[1]	Ayrshire	1	>200	0.23 to 0.36
			>600	0.59 to 0.93
		2	>200	0.53 to 1.25
			>600	1.36 to 3.23
(Halasa et al., 2009)[2]	Holstein	1	>200	0.20 to 0.35
		2	>600	0.44 to 0.56
(Hagnestam-Nielsen et al., 2009)[2]	Swedish Holstein and Swedish Red	1	>200	0.1-1.2
			>500	0.7-2.0
			>1000	1.2-2.6
		>1	>200	0.9-2.2
			>500	1.1-3.7
			>1000	2.1-4.8
(Hand et al., 2012)[1]	Holstein	1	>200	0.35
			>600	0.89
			>1000	1.15-2.67
		2	>200	0.49
			>600	1.28
			>1000	2.04-3.56

[1] Loss in daily milk expressed as deviation from milk yield of cows of the same parity having SCC <100 $\times 10^3$
[2] Loss in daily milk expressed as deviation from milk yield of cows of the same parity having SCC <50 $\times 10^3$

In addition, some evidence was found on a residual effect of mastitis, both clinical and subclinical, on the milk production for the rest of the lactation as well as for the subsequent lactations. After being diagnosed with a CM at week 3 or 6, cows produced less milk than healthy cows throughout the rest of the lactation (Hagnestam et al., 2007). Cows with CM did not regain their pre-mastitis milk yields and yielded between 0.7 to 2.5 kg/d less milk (depending on parity) for the rest of the lactation than they would have without mastitis (Rajala-Schultz et al., 1999). Cows with 3 clinical quarters or more during their first lactation had 381 kg less milk during the second lactation (Houben et al., 1993). For third lactation milk production, the effect of increased SCC during the second lactation was about 20 to 30% as much as the effect of SCC increase in the third lactation (Fetrow et al., 1991).

1.2.3. Mastitis and reproduction

The highest incidence of mastitis is observed in early lactation, and many studies reported a negative association between mastitis and reproductive performance. The hypothesis of a negative impact of mastitis on the reproductive cycle via the release of bacterial endotoxins that disrupts the secretion of inflammatory mediators such as prostaglandin PGF2α and the secretion of Luteinizing hormone (LH) and estradiol is already evocated (Risco et al., 1999; Santos et al., 2004; Suzuki et al., 2001). The infectious diseases could cause anovulation, fertilization failure and embryonic mortality (Peter, 2004). Mastitis incidence was associated with an increase in the number of days to first artificial insemination (AI1) (Barker et al., 1998) and a greater number of services per conception (Moore et al., 1991) with a higher risk of abortion (Risco et al., 1999; Santos et al., 2004). Such effect has been reported in cows with SCM or CM. Genetic correlations between mastitis diagnosed by the SCC and reproduction traits ranged from 0.27 to 0.33 (Pritchard et al., 2013) and from 0.21 to 0.41 (Kadarmideen et al., 2000). The strength of the association depends on the severity, the duration and the timing of the mastitis for both clinical and subclinical forms, as detailed below.

Clinical mastitis occurring from 14 d before to 28 d after artificial insemination (AI) was associated with decreased conception rates (CR) (Hertl et al., 2010). A wider time frame (from 28 days before to 70 days after relative to the risk period of a cow becoming pregnant) was reported (Hudson et al., 2012). CR were lower for cows with CM (38%) than uninfected cows (46%) when CM event was evaluated within 30 days post AI (Kelton et al., 2001). Cows with CM within the first 45 days of gestation were shown to be at greater risk (Odds ratio OR [and 95% CI] = 2.7 [1.3-5.6]) of abortion within the next 90 days than uninfected cows (Risco et al., 1999). Similarly, cows with CM within the time frame of the day of AI to pregnancy reconfirmation were 2.8 times more likely (OR [and 95% CI] = 2.8 [1.2-6.8]) to lose their pregnancy than those not experiencing mastitis (Chebel et al., 2004). Lastly, cows with CM have more days open (107 ± 5 *vs*. 88 ± 2, $P < 0.05$), more services per conception (2.1 ± 0.1 *vs*. 1.6 ± 0.1, $P < 0.05$) and lower CR (48% *vs*. 63%, $P < 0.05$) compared to cows without mastitis (Frago et al., 2004). These results were confirmed by (Santos et al., 2004): cows with CM prior to AI1 or between AI1 and pregnancy confirmation had a greater ($P < 0.05$) number of days non-pregnant (165.0 and 189.4 days), decreased CR (22.1 and 10.2%) , and greater incidence of abortion (11.8 and 11.6%) compared with uninfected cows (139.7, 28.7% and 5.8%), respectively.

These results suggest that, although it appears that CM either prior or after the first postpartum AI impairs the reproductive performance of dairy cows, the occurrence of mastitis after the AI1 sounds to have a more dampening effect on the reproductive performance than before the AI (Loeffler et al., 1999a; Santos et al., 2004). The number of AI per conception and days to conception were significantly ($P < 0.01$) greater for cows with CM after AI1 (2.9 and 136.6) than for cows with CM before AI1 (1.6 and 113.7) or cows with CM after confirmed pregnancy (1.7 and 92.1), respectively (Barker et al., 1998). Similarly, CM has an extremely weak effect on conception (OR [95% CI] = 0.9 [0.69-1.30], $P = 0.74$) if occurring prior to AI and is associated with > 50% reduction in pregnancy risk (OR [95% CI] = 0.44 [0.20-0.98], $P < 0.05$) if occurring in the 3 weeks just after AI (Loeffler et al., 1999a).

The negative association between SCM and reproduction performance is also consensual. Cows with SCM (diagnosed by bacteriological sampling) have longer (74.8 vs. 67.8, $P < 0.01$) interval between calving and first service (Schrick et al., 2001). Compared with uninfected controls, cows with chronic moderately (between 150,000 and 450,000 cells/mL) and highly (between 450,000 and 10^6 cells/mL) elevated SCC at the test-dates before and after the breeding have their probability of conception decreased by 14.1 to 14.5% ($P < 0.05$). However, the very highly elevated SCC (> 10^6 cells/mL) subgroup of subclinical cows exhibited the largest decline in the probability of conception (20.5%, $P < 0.05$) (Lavon et al., 2011b). Cows with an increase of SCC to > 200,000 cells/mL after calving had a lower probability (OR [95% CI] = 0.40 [0.38-0.42], $P < 0.0001$) of pregnancy at AI1 and had a higher number of AI per animal submitted for AI than healthy cows (Lomander et al., 2013). Change (increase) in the SCC around AI is clearly identified as influencing conception. For instance, cows with chronic SCM (increased SCC at the test-dates before and after the breeding) had a 14.3% decrease in probability of conception as compared with uninfected or cured cows (Lavon et al., 2011b). Moreover, increased test-day SCS lengthened ($P < 0.01$) both calving to first service and calving to conception intervals by 1.3 to 2.0 days for each unit increase in SCS (Rekik et al., 2008). Mean calving to AI1 interval was prolonged by 11.7 days ($P < 0.01$) in cows with SCM (Klaas et al., 2004). Lastly, cows with CM or SCM before first service had increased days to first service (77.3 and 74.8), days open (110.0 and 107.7), and services per conception (3 and 2.1) compared with controls (67.8, 85.4 and 1.6), respectively (Schrick et al., 2001). However, the most deleterious effect is observed when SCM progressed to acute clinical case: cows initially diagnosed with a subclinical infection that became clinical during the period between first service and pregnancy exhibited significantly increased days to first service (93.9), days open (196.0), and services per conception (4.3) compared with control animals ($P < 0.05$). Typical for clinical events, (Lavon et al., 2011a) reported that a single elevation in SCC after AI had a more prolonged dampening effect on the probability of conception than before AI.

Compared with healthy cows, OR of pregnancy [95% CI] were 0.56 [0.33-0.94], 0.67 [0.49-0.92], and 0.75 [0.57-0.97] for cows experiencing chronic CM, CM, or SCM during the breeding risk period, respectively (Fuenzalida et al., 2015). Calving to conception intervals were 119.1, 141.7 and 94.1 for the last mentioned groups, respectively.

1.2.4. Underlying mechanisms linking reproduction and mastitis

Mechanisms implicated in the negative association between mastitis and reproductive performance remain speculative, but several hypotheses are proposed. It is thought that mastitis occurrence in the early lactation may impair the cow's ability to show estrus (thus, reducing detection) and to conceive after AI (Pritchard et al., 2013).

First, the increase in body temperature associated with mastitis damages reproductive processes that may interact with oocyte and embryo function (Edwards and Hansen, 1997).

Second, mastitis may decrease the feed intake and alter metabolite concentrations, resulting in changes of the hormonal profile of the cow and inhibiting follicular development (Oliver et al. 2000; Young 2000).

Third, mastitis could affect the resumption of ovarian activity in post-partum cows: cows with mastitis at 15 to 28 DIM had delayed luteal activity and reduced estrous behavior (Huszenicza et al., 2005). This may involve alteration of the inter-estrus intervals and disruption of LH secretion, leading to delayed ovulation, which can explain the reproductive failure associated with this disease (Moore et al., 1991; Lavon et al., 2011b). This extension of the estrus-to-ovulation interval from about 30 to 60 h may reduce the fertilization success because of the short fertile life of spermatozoa in the female tract and of the oocytes after ovulation (Hunter, 1994).

Fourth, the mastitis-associated inflammation releases cytokines, which may inhibit the action of FSH on LH receptor formation, and alter their activity and functions (Darbon et al., 1989). The oocyte maturation and fertilization may be affected (McCann et al., 1997; Soto et al., 2003). Endotoxin, a component of the cell wall of Gram-negative bacteria that causes an inflammatory response, might induce luteolysis and influence conception and early embryonic survival by the release of inflammatory mediators (Cullor, 1990). The production of theses bioactive molecules can potentially alter the reproductive tract functions by causing luteal regression (Moore and O'Connor, 1993), then disrupting progesterone secretion, endometrial functions, and embryonic development (Gilbert et al., 1990; Spencer et al., 2004).

1.3. Hyperketonemia in dairy cows

1.3.1. From negative energy balance to hyperketonemia

The efficient nutrient management of dairy cattle around calving is one of the most important key factors in successful dairy farming, taking into account its influence on both milk production and reproduction. This transition period is meanly composed of two stages that are very different: first, the end of the dry-off period characterized by low dietary needs, and second the early lactation characterized by high needs of nutrients, in particular energy. As a result, cows' nutrient requirements increase dramatically after calving, but at the same time, ingestion of the cow is limited. A reduced dry matter (DM) intake is usually observed during the last month of gestation, more specifically in the last week before calving in dairy cows (Hayirli et al., 2002). The increase of DM intake after calving is slower than the increase in nutrient needs for lactation. From the calving up to around the lactation peak –and often later- high yielding cows are unable to ingest a sufficient amount of diet, to support the required energy for maintenance and milk production. Cows requirements of energy and protein in the first two weeks of lactation have been reported to exceed those available in the diet by 26 and 25%, respectively (Bell and Bell, 1995). Furthermore, respectively 97% and 83% of energy and protein obtained are utilized by the udder limiting coverage of maintenance needs (Drackley, 1999). The gap between intake and needs may start few days before calving due to the drop in DM intake and increased needs for late gestation growth of the fetus. As a result, cows use body reserves of accumulated lipids as an energy source: they often lose 60% or more of their body fat in the first weeks after parturition and as a consequence will lose body condition in the process (Wright and Russel, 1984).

The difference between diet energy supply and requirements for maintenance and milk synthesis is commonly called the energy balance (EB). It is often negative after calving and during early lactation in mammals (Robinson, 1986). Excessive NEB can lead to several negative events (deterioration of milk production and reduced reproductive efficiency), beside numerous postpartum diseases. The longer the nadir of the calculated NEB (cNEB) lasts, the longer and deeper the NEB will be (Jorritsma et al., 2003). The fat mobilization is characterized by a release of free non-esterified fatty acids (NEFA) and the production of the major ketone bodies, acetone, acetoacetate and beta-hydroxybutyrate (BHBA) (Hungerford, 1990).

Hyperketonemia (also known as ketosis) is defined as an increased concentration of ketones in blood. The increase in these compounds concentration in the blood of dairy cows after calving is a consensual indicator of NEB after calving, and they are commonly used to diagnose clinical (CK) and subclinical ketosis (SCK) (Andersson, 1988; Drackley, 1999; Herdt, 2000). CK can manifest as a decrease in appetite, weight loss and a decrease in milk production. In addition to ketones, NEFA is also another precious metabolite used in practical to diagnose this condition. Low blood glucose, low blood insulin and low blood insulin-like growth factor I (IGF-I) (Ingvartsen and Andersen, 2000) may also be used, but they face specific challenges in the field. In addition to hyperketonemia, the excessive mobilization of stored body fat needed as an energy source can result in excessive accumulation of fat in the liver leading to fatty liver syndrome (Goff and Horst, 1997).

1.3.1.1. Prevalence and importance of hyperketonemia

The highest incidence of hyperketonemia is reported within the first 1 to 3 weeks of lactation (Oetzel, 2004; Duffield et al., 2009). In Holstein cows, 87% of ketosis cases are recorded during the first 30 days of lactation (Koeck et al., 2013). The incidence of SCK starts remarkably soon after calving with a peak at 5 DIM (McArt et al., 2011). The incidence rates of CK was reported to be between 2 and 15% (Duffield, 2000). A recent European study showed within-herd prevalence of ketosis (defined as milk BHBA ≥ 100 μmol/L) of 53% in France, 43% in Germany, 31% in Italy, 46% in the Netherlands, and 31% in the United Kingdom (Berge and Vertenten, 2014). The incidence rates of SCK (defined as blood BHBA ≥ 1.2 m*M*) is estimated to range from 20 to 60% (Duffield et al., 1998). More recently, the incidence of SCK (defined as blood BHBA concentration of 1.2 to 2.9 m*M*) in the USA was reported to be 43% (ranging from 26 to 56%) with a peak incidence (28.9%) occurring at 5 DIM (McArt et al., 2012).

Recent results identified the total cost of SCK. The total cost per case of SCK was estimated at $375 and $256 for primiparous and multiparous animals, respectively, with an average total cost of $289 per case (McArt et al., 2015). Even a close estimation was found by (Raboisson et al., 2015), they reported that this estimation does not seem sufficiently accurate because it is based on raw data which might result in a total cost substantially overestimated. They also reported that excluding labor costs in the estimation reduced the SCK total cost by 12%, whereas excluding contributors with scarce data and imprecise calibrations (for lameness and udder health) reduced costs by another 18–20% (€210, 95%PI = 30-390).

1.3.1.2. Risk factors of hyperketonemia

Hyperketonemia prevalence is influenced by a variety of factors such as body condition score (BCS), parity, season and herd-related factors. Higher BCS at the end of the dry period is a key risk factor for SCK. High BCS reduces dramatically DM intake after calving, and consequently leads to a more severe NEB (Garnsworthy and Topps, 1982). Several studies showed that the cNEB depends to a larger extent on the increase of feed intake postpartum (Butler et al., 1981; Villa-Godoy et al., 1988). Other endocrinology and metabolic characteristics of cows could play an important role in the NEB intensity (Jorritsma et al., 2003). Cows with a moderate to high BCS before calving were more likely to develop SCK and CK than thin cows (Hayirli et al., 2002; Vanholder et al., 2015). Prolonged previous lactation length and dry period length were both associated with increased odds for SCK and CK (Vanholder et al., 2015; Berge and Vertenten, 2014). This may be related to higher BCS in these cows. In a recent meta-analysis (Raboisson et al., 2016), the association between SCK and BCS was reported in 6 models from 3 publications, all adjusted for parity. The mean relative risk (RR) [95% CI] of SCK for BCS > 3 compared to BCS < 3 was 1.27 [0.92-1.77]. The mean RR [95% CI] of SCK for BCS > 4 compared to BCS < 3 was 1.63 [1.04-2.56]. In the same meta-analysis, the association between SCK and parity was evaluated using 12 models from 5 publications. The mean RR [95% CI] of SCK for parity > 1 compared to parity = 1 was 2.22 [1.72-2.84]. The mean RR [95% CI] of SCK for parity = 3 compared to parity = 2 was 2.82 [1.87-4.10].

High milk-producing cows were generally found to be genetically more susceptible to ketosis (Lyons et al., 1991; Uribe et al., 1995). Calving during the hot and cold seasons has been reported as a risk factor for ketosis compared to moderate ones (Andersson and Emanuelson, 1985; Gröhn et al., 1989; Berge and Vertenten, 2014). Larger herd size was associated with a decreased risk of ketosis and farms that fed partially mixed rations had 1.5 times higher odds of ketosis (OR [95% CI] = 1.5 [1.1-2.1], $P < 0.01$) than those that fed total mixed rations (Berge and Vertenten, 2014). Increased dry period length and transition cow feed management have also been associated with an increased ketosis risk (Duffield et al., 1997; Berge and Vertenten, 2014).

1.3.1.3. Consequences of hyperketonemia

The associations of hyperketonemia or SCK and several disorders or diseases have been recently gathered in a meta-analysis (Table 3). The meta-analysis of the literature included 131 different models from 23 papers and adjusted the RR or OR by the definition of hyperketonemia or SCK and by the covariates included in the models of the available papers. Hyperketonemia or SCK were defined as (i) BHBA > 1.4 m*M* postpartum, (ii) NEFA > 0.4 m*M* prepartum or (iii) NEFA > 1.0 m*M* postpartum It clearly shows that many key items of peripartum dairy cow health or production are associated with hyperketonemia.

Table 3. Risk of different peripartum disorders in cases of subclinical ketosis obtained via a meta-analysis (Raboisson et al., 2014b)

Peripartum disorder	Data included Studies no.	Models no.	Outcome of interest	Adjusted value[1] *[95% CI]*
Displaced abomasum	10	38	RR	3.3 *[2.6-4.3]*
CK	5	16	RR	5.4 *[3.2-8.8]*
Early Culling (< 60 days)	3	10	RR	1.9 *[1.6-2.3]*
Metritis	5	12	RR	1.8 *[1.5-2.0]*
Retained placenta	3	3	RR	1.5 *[1.2-1.9]*
Endometritis	1	2	RR	1.4 *[1.1-2.0]*
Clinical mastitis	3	4	RR	1.6 *[1.2-2.1]*
×2 increase in SCC	2	4	RR	1.4 *[1.3-1.6]*
Lameness	1	3	RR	2.0 *[1.6-2.5]*
Milk yield	4	13	Liters *(sd)*	- 251 *(73)* / -112 *(89)*
Calving rate at AI1	2	5	RR	0.67 *[0.53-0.83]*
Prolonged calving–calving interval	3	6	Days	21

[1]Values of risk adjusted for the effect of covariates.

1.3.1.4. Diagnosis of hyperketonemia

Hyperketonemia is highly suspected on high BCS decrease and the definitive diagnosis relied on high BCS decrease and on blood and milk biochemistry. Increased concentrations of both BHBA and NEFA are used as markers of SCK. The concentration of serum NEFA correlates with the magnitude of fat mobilization, whereas the concentration of BHBA reflects the incompleteness of fat oxidation in the liver (LeBlanc, 2010a). NEFA is then recognized as an earlier indicator of NEB compared to BHBA and can be used before or after calving whereas BHBA remains useful only after calving. The BHBA threshold of 3m*M* has been proposed for CK but this should be associated with clinical signs such as anorexia and depression (Oetzel, 2004; McArt et al., 2011). SCK is consensually defined as a blood BHBA greater than 1.4 m*M* during early lactation (Duffield et al., 2009). This definition is considered as the gold standard. Although a few publications have reported other thresholds for BHBA to diagnose SCK (Table 4), the threshold has generally been acknowledged to be between 1.2 and 1.4 m*M* (Raboisson et al., 2014).

Table 4. Different NEFA and BHBA thresholds used for the diagnosis of subclinical ketosis from (Ospina et al., 2010b) and (Oetzel, 2004)

Period of detection	Evaluated criteria	Test	Critical threshold	Herd alarm level (%)
Prepartum	Displaced abomasum, CK	NEFA	0.27 mEq/L	15
	Pregnancy rate	NEFA	0.27 mEq/L	15
	305-day milk yield	NEFA	0.27 mEq/L	15
Postpartum	Displaced abomasum, CK	BHBA	1.2 m*M*	15
	Pregnancy rate	BHBA	1.2 m*M*	15
	305-day MY (Parity 1)	BHBA	1.2 m*M*	20
	305-day MY (Parity > 1)	BHBA	1.0 m*M*	15
Postpartum	Displaced abomasum, CK	NEFA	0.70 mEq/L	15
	Pregnancy rate	NEFA	0.60 mEq/L	15
	305-day MY (Parity 1)	NEFA	0.60 mEq/L	15
	305-day MY (Parity > 1)	NEFA	0.70 mEq/L	15

The results of this meta-analysis showed that using 1.0, 1.2, or 1.4 m*M* interchangeably as a BHBA threshold could result in important over- or underdiagnoses. Although postpartum BHBA values have been used as a recognized gold standard for diagnosing SCK, NEFA values also appear to be a useful tool. The metabolite NEFA is commonly used before calving (Oetzel, 2004). However, although prepartum NEFA and postpartum BHBA were both significantly associated with development of clinical disease, postpartum serum NEFA concentration was most associated with the risk of developing displaced abomasum, CK, metritis, or retained placenta during the first 30 d in milk (Ospina et al., 2010b).

Interestingly, the publications analyzing the relationship between SCK and various disorders defined simultaneously the threshold to define SCK and the value of OR, RR or hazard ratio (HR) relative to the disorder studied. The calculated OR, RR or HR have been highly dependent on the defined BHBA and NEFA thresholds, and these thresholds have not always matched the chosen definition of SCK. In most cases, several thresholds have been tested, and the best model (lowest P-value) has been retained (with the best threshold and OR, RR, or HR linked). The same issue was faced when the alarm herd-level has to be defined, as shown in Table 4. The outcome studied, the test used and the threshold retained interact to define the best combination of parameters to highlight an issue at the herd level. However, for practical purposes, the chosen definition of SCK and the herd-level prevalence alarm has been assumed to remain constant. Table 5 shows how the combination of parameters, defined as expert opinion or by epidemiological tools, may lead to close situations (higher threshold and lower alarm and vice versa).

Table 5. Combination of different biochemical parameters to define subclinical ketosis (SCK)

Test	Detection period (days to calving)	Critical threshold	Herd alarm level (%)	Reference
BHBA	5 to 60	> 1.2 m*M*	15	(Ospina et al., 2010b)
		> 1.4 m*M*	10	(Oetzel, 2004)
NEFA	-14 to -2	> 0.3 mEq/L	15	(Ospina et al., 2010b)
		> 0.4 mEq/L	10	(Oetzel, 2004)
NEFA	5 to 30	> 0.6 mEq/L	15	(Ospina et al., 2010b)

The milk composition can also reflect the energy status of the animal. The milk fat and protein contents were proposed as indicators to evaluate nutritional problems, in particular energy deficit in lactating dairy cattle. Ketosis is known to be associated with increased milk fat content due to adipose tissue mobilization and decreased milk protein content due to a shortage of glucose for milk protein synthesis in the udder (de Vries and Veerkamp, 2000). Thus, observing fat and protein contents in the milk may offer an easily measurable alternative to evaluate the energy status since it can be obtained from routine milk testing (Heuer et al., 1999). A genetic correlation of 0.63 was found between metabolic diseases and fat : protein ratio (FPR) in early lactation based on data from a research farm (Buttchereit et al., 2012).

Mean test-day FPR varies widely for different breeds and during different stages of lactation. The highest values were recorded at the beginning of lactation but then stabilized over the lactation with reported values ranging between 1.1 and 1.45 during lactation (Buttchereit et al., 2010; Jamrozik and Schaeffer, 2012; Negussie et al., 2013). The optimal FPR reported by (Gravert, 1991) is of 1-1.25, whereas (Duffield et al., 1997) sets 1.33 as a high margin. Doing research on Holstein cows, (Čejna and Chládek, 2005) have indicated that the optimum FPR was between 1.2 and 1.4. (Heuer et al., 1999) Results show that cows with a FPR > 1.5 in their first test-day milk record had higher risk for ketosis. FPR lower than 1 is considered at risk of subacute ruminal acidosis (Eicher, 2004). Altogether, FPR stays an interesting tool for hyperketonemia identification, among others, due to its availability on all cows, but this mean suffers for moderate accuracy, with many false positive or false negative diagnosed animals, and then an imprecise prevalence definition. The sensitivity and the specificity of test-based on the evaluation of fat, protein and their ratio are summarized in Table 6.

Table 6. A summary of the sensitivity (SN), specificity (SP) of tests based on fat and protein contents in milk to identify hyperketonemic cows

Test	DIM[1]	Gold standard: BHBA (m*M*)	SN	SP	Reference
FPR[2] > 1.5	< 65	1.2	0.22	0.85	(Duffield et al., 1997)
	< 65	1.2	0.66	0.71	(van Knegsel et al., 2010)
	7-21	//	0.63	0.79	(Krogh et al., 2011)
FPR > 1.35	< 65	1.2	0.58	0.69	(Duffield et al., 1997)
Fat ≥ 41 mg/L	< 65	1.2	0.55	0.85	(Duffield et al., 1997)
Protein ≤ 28 mg/L	< 65	1.2	0.50	0.80	(Duffield et al., 1997)

[1]Days in Milk

[2]Fat: Protein ratio

1.3.2. Hyperketonemia and milk production

The difficulties in analyzing the association between milk production and SCK can be summarized as follows: the higher the ketones are during early lactation, the higher the milk losses will be, but the greater the cow's production is, the greater risk she has of succumbing to ketosis, with milk drop induced. A recent review investigated the relationship between milk production and SCK defined through BHBA and NEFA concentrations (Raboisson et al., 2014b). It highlighted that the association was often negative when SCK was diagnosed during early lactation and that it depended on parity (Table 7). The overall direct and indirect mean (±sd) 305-d milk loss associated with SCK was 340 (±48) kg of milk. When subtracting the milk losses directly due to all disorders and diseases associated with SCK, the direct average milk loss (sd) related to SCK was 112 (±89) kg, or 251 (±73) kg, if adjustments were made for abomasal displacement, CK, metritis and placental retention.

Table 7. A summary of the relationship between subclinical ketosis (SCK) and milk production reported in literature; source: (Raboisson et al., 2014b)

Reference	Parity	Prevalence of SCK (%)	Diagnosis of SCK		Difference in milk production if SCK		
			Test: threshold (m*M*)	Week (no.) of detection[1]	Value (kg)	P-value	Definition[2]
(Ospina et al., 2010a)							
	All		NEFA: 0.33	−2 to −1 (1)	−683	< 0.001	ME 305-d production calculated at 120 DIM
	1		NEFA: 0.57	+1 to +2 (1)	488	0.02	
	1		BHBA: 0.9	+1 to +2 (1)	403	0.4	
	>1		NEFA: 0.72	+1 to +2 (1)	−647	0.001	
	>1		BHBA: 1.0	+1 to +2 (1)	−393	0.04	
(Duffield et al., 2009)							
	All	16	BHBA: 1.4	+1 (1)	−126	0.33	305-d production calculated at third test
	All	12	BHBA: 1.8	+1 (1)	−333	0.04	
	All	24	BHBA: 1.2	+2 (1)	272	0.008	
	All	16	BHBA: 1.4	+2 (1)	236	0.06	
(McArt et al., 2013)							
	All	43	BHBA: 1.2 to 2.9	+1 and +2 (6)	−1.2	0.006	Daily milk (0-30 DIM)
	All	43		+1 and +2 (3)	−2.1	0.04	
(Dohoo and Martin, 1984)							
	All	12	BHBA milk	+1 to +4 (1)	−1.03[3]	< 0.05	Daily milk (< 65 DIM)
	All	5	BHBA milk	+1 to +4 (1)	−1.40[4]	< 0.05	
(Ospina et al., 2010c)							
	All	>15	NEFA: 0.27	−2 to −1	−282	< 0.001	ME 305-d production
	1	>15	NEFA: 0.6	+1 to +2	−288	< 0.001	
	>1	>15	NEFA: 0.7	+1 to +2	−593	< 0.001	
	1	>20	BHBA: 1.2	+1 to +2	−534	< 0.001	
	>1	>15	BHBA: 1.0	+1 to +2	−358	< 0.001	

[1] Peripartum week of detection (and number of detections).
[2] ME = mature equivalent.
[3] Corresponds to −4.4%.
[4] Corresponds to −6.0%.

1.3.3. Hyperketonemia and reproductive performance

The negative association between NEB or ketosis and reproductive performance is consensual, based on pathophysiological and epidemiological evidences, despite many controversies for the epidemiological results. NEB has been shown to have a significant influence on reproductive performance of dairy cows (Wathes et al., 2003). The link between the magnitude and duration of NEB and the reduced health and reproductive performance has been widely investigated through phenotypic or environmental correlations (Harrison, 1990; Waltner et al., 1993; Derouen et al., 1994). In addition, a few studies indicate a genetic correlation between EB and fertility. For example, a positive EB with higher body weight during lactation had an unfavorable genetic correlation (−0.40 to −0.80) with days until first luteal activity (Veerkamp et al., 2000).

The associations between SCK and reproduction have been extensively studied in 2 recent meta-analysis (Raboisson et al., 2014b; Abdelli et al., 2017). Results of both studies were in agreement (Tables 3 and 8). Both concluded to the scarcity of studies, low level of evidence and low precision of the quantifications. The OR or RR [95% CI] for placental retention, metritis and pregnancy at first AI in cases of SCK were 1.5 [1.2-1.9], 1.9 [1.7-2.1] and 0.62 [0.41-0.93], respectively (Table 8). The HR [95% CI] for time to pregnancy in cases of high NEFA or BHBA was 0.77 [0.61-0.97]. No conclusion can be done for estrus cyclicality, with positive or negative association depending on the studies.

Table 8. Results from meta-regression of the effects of elevated NEFAs and BHB on several reproduction traits and disorders; source: (Abdelli et al., 2017).

Reproductive disorders	Risk (95% CI)	*P*-value
Metritis	1.91 (1.72 - 2.12)[1]	< 0.0001
Placental retention	1.51 (1.19 - 1.92)[1]	< 0.001
Pregnancy at AI1	0.62 (0.41 - 0.93)[1]	< 0.0001
Time to pregnancy	0.77 (0.61 - 0.97)[2]	< 0.05
Estrous cyclicality	1.14 (0.99 - 1.29)[1]	< 0.10

[1] risk is expressed as relative risk
[2] risk is expressed as hazard ratio

The duration of exposure to elevated circulating BHBA has been demonstrated to be determinant in its association with reproduction. Cows identified as SCK in either of the first 2 weeks were 17% less likely (OR [95%CI] = 0.83[0.160-1.15]) to be pregnant after AI1, whereas cows that remained above the SCK thresholds in both weeks were 53% less likely (OR [95%CI] = 0.47 [0.29-0.77]) to be pregnant (Walsh et al., 2007). The association may also be time dependent: cows first diagnosed with SCK between 3 and 7 DIM were 30% less likely (OR [95%CI] = 0.7 [0.6-0.8]) to conceive at first service compared to cows first testing positive between 8 and 16 DIM (McArt et al., 2012).

These associations have also been investigated through the fat and protein contents. The effects on pregnancy risk of extremely large changes in FPR were substantial (OR [95%CI] = 0.49 [0.33-0.72] and 0.77 [0.65-0.92] for a deviation of > 0.4 and 0.2 to 0.4 from the herd mean, respectively) during early lactation (Loeffler et al., 1999a). Similarly, a positive and significant (P < 0.05) genetic association in early lactation between FPR and days to AI1 or days open (0.28 and 0.24; respectively) were reported (Negussie et al., 2013).

The association between CK and reproduction is also reported. For instance, cows with CK had significantly (P < 0.05) longer days open (139 vs. 85 days) and greater culling rate due to a failure to conceive (Cook et al., 2001). Table 9 summarizes the results of a meta-analysis (Fourichon et al., 2000) using ten studies to reveal the association between CK and reproductive parameters in dairy cows.

Table 9. Effects of clinical ketosis on reproductive performance in dairy cows as reviewed by (Fourichon et al., 2000) explained as deviation from cows without clinical ketosis

Parameters	Summary estimate	Summary 95% CI
Days to first service	+2.5	1.0 - 4.1
Occurrence of first service	NS	-
Conception at first service (%)	-3.8	-8.0 - 0.5
Interval between AI1 and conception	+4.7	1.9 - 7.4
Days open in cows conceiving	+5.9	3.0 - 8.8
Conception from 56 to 120 DIM (HR)	0.87	0.82 - 0.91
Daily probability of conception (HR)	NS	-
Services per conception	+0.13	0.06 - 0.19

1.3.4. Underlying mechanisms

The underlying mechanisms related to the association between NEB or SCK and reproductive performance are extensively described, even if the weight and the importance of some of the various mechanisms involved remain unclear. The main steps of a successful reproduction are influenced by NEB or SCK (Rossi et al., 2008): dominant follicle selection, suitable hormonal environment before ovulation in particular on primary follicles, biochemical environment before ovulation in particular on dominant follicles (follicular fluid), fecundation and spermatic characteristics, status of uterine tract to received oocyte.

First, a negative effect of early lactation conditions on the oocyte quality has been reported (Snijders et al., 2000; Walters et al., 2002). Postpartum NEB has significantly impaired the quality of oocytes offered for fertilization 80–100 days and required for follicle development, resulted in reducing CR (Britt, 1992).

Second, primary follicle is highly sensitive to hormonal environment. Metabolic hormonal modifications regulated by pituitary-hypothalamic axis (as LH, FSH, GH, insulin, leptin, IGF-1, estrogen and progesterone) are observed during NEB. LH pulsatility has been affected by postpartum status of EB (Canfield and Butler, 1990). Pulsatile LH secretion is one of the driving hormones for ovarian follicular development and ovulation (Schillo, 1992). Low blood glucose is believed to delay first ovulation by decreasing LH pulsatility (Jorritsma et al., 2003). NEB damage on LH secretion and ovulation has been confirmed by (Beam and Butler, 1997) who reported that follicles emerging after the NEB nadir have more possibility to ovulate than follicles emerging before. During the first two weeks post-calving there is a profound decrease of IGF-1 plasmatic concentrations, which decrease the follicle capacity to produce required estrogens for next ovulation (Butler, 2003), and this influences directly several reproductive parameters (calving to first ovulation and to conception intervals).

NEB has also been associated with dramatically reduced insulin and IGF-I concentrations (Spicer et al., 1990). Their enhanced role on the ovarian activity and follicle cell proliferation is already known (Wathes, 2012). This negative effect could also be due to a reducing progesterone secretion after calving (Villa-Godoy et al., 1988; Spicer et al., 1990).

Numerous studies reported a delay from calving to first service compared with cows without NEB, limiting the number of estrus cycles occurring before the AI1 (Butler et al., 1981; Wathes et al., 2007b). Each decrease of 10 MJ of net energy per day in nadir of EB was associated with a 1.25 day longer interval in the postpartum start of the luteal activity and the first ovulation (de Vries and Veerkamp, 2000). Reducing this interval provides more ovarian cycles prior to AI and consequently improves the CR (Butler and Smith, 1989). The timing of the NEB nadir has been also implicated in the interval to first ovulation (Beam and Butler, 1999) that occur about 30 days (range of 17–42 days) postpartum (Butler and Smith, 1989; Staples et al., 1990). All ketone bodies, milk acetone and serum BHBA concentrations provided the most reliable information with regard to resumption of ovarian activity (Reist et al., 2000).

Third, metabolic changes associated with NEB after calving have altered the composition of follicular fluid and oocytes maturation and fertilization in vitro (Leroy et al., 2005), although this is not constantly observed (Matoba et al., 2012). The changes of BHBA and NEFA concentrations in serum were reflected by similar changes in follicular fluid (Leroy et al., 2004), especially for BHBA where the correlation was always significant up to 46 days postpartum.

During the transition period, plasma concentrations of NEFA and BHBA and hepatic accumulation of triglycerides were higher for cows in which the first postpartum dominant follicle failed to ovulate in comparison with cows that had ovulatory follicles (Marr et al., 2002).

In vitro experiment showed that oocytes maturation was delayed in the presence of NEFA, which caused a delay in the oocytes fertilization. Cleavage, and embryonic development after maturation were significantly reduced (Jorritsma et al., 2004). A direct toxic effect of high NEFA and low glucose concentrations on oocyte maturation has been suggested, and an additive toxic effect might be induced by BHBA in subclinical conditions (Leroy et al., 2006). NEFA was reported to modulate granulosa cell proliferation and steroidogenesis in vitro, which could be involved in the occurrence of ovarian dysfunction during the postpartum period in high-yielding dairy cows (Vanholder et al., 2005).

Fourth, optimum progesterone level is essential to maintain the luteal support for uterus (Butler et al., 1996b). Cows with a severe NEB were shown to have reduced progesterone secretion that impaired the luteal support for the uterus during pregnancy, and thereby lower the CR (Spicer et al., 1990).

Fifth, the relationship between uterine functionality and immune response during pregnancy and transition period has been highlighted. Cows experiencing NEB prior to or around calving are exposed to periparturient immune suppression (Hammon and Goff, 2006). The impairment of uterine leukocytes and polymorphonuclear cells (PMN) is particularly observed although they play an important role as a first line of cellular defense against bacteria colonization within the uterus (Rhoads et al., 2006). This could alter the immune response to uterine inflammation which occurs after calving whether or not a cow will develop metritis (Wathes et al., 2009). The shortage of recovery rate represented by subclinical endometritis has been associated with delayed onset of ovarian cycles and longer pregnancy intervals and lower CR (Galvao et al., 2009).

Both the level of impairment and the rate of recovery of immune capabilities at postpartum are strongly influenced by the extent of NEB around calving (Wathes et al., 2009). Also, suppression of phagocytic activity of macrophages by ketones has been reported (Klucinski et al., 1995).

Reproductive performance can also be impaired due to uterine disease and delayed luteal activity, which in turn are associated with elevated concentrations of NEFA and BHBA (Hammon et al., 2006; Wathes et al., 2007a).

1.4. Nitrogen imbalance and reproduction

1.4.1. Definition and risk factors

Protein supply is another challenge in ration adjustment in the early lactation period. Dairy cows need protein intake for milk synthesis, immune system, growth and pregnancy. The protein requirements could be achieved from dietary protein, rumen microbial crude protein (CP), and from the mobilization of body tissue. Rumen bacteria require a source of nitrogen to grow. If there is inadequate nitrogen in the diet, microbial growth and subsequent digestion of food will be impaired (NRC, 1985, 2001).

During NEB, body fat mobilization results in proportionally more available energy than protein in the body. Therefore, the dietary protein intake during early lactation period should be increased to maximize the efficiency of energy utilization and milk production (Kung and Huber, 1983; Grings et al., 1991). Risk of nitrogen imbalances is then high in this period, but it can also occur later in lactation due to accidental imbalances.

Increasing dietary protein intake is one of the followed strategies to enhance milk production, but those diets could exceed cows requirements for rumen degradable (RDP) or undegradable protein (RUP), and this excess can contribute to impaired reproductive performance of dairy cows (Butler, 1998). That could explain, partly, the negative correlation between high milk production and the reproductive efficiency observed by several studies (Butler, 1998; Lucy, 2001; Pryce et al., 2004).

Through normal ruminal fermentation, RDP provides a source of amino acids, ammonia and nucleic acids for rumen microbial protein synthesis (Butler et al., 1996a). The Excessive RDP or RUP supplementation in lactational rations associated with relative shortage of energy to synthesize bacterial proteins may result in higher concentrations of ammonia in rumen and blood (Lobley et al., 1995). In addition, higher postpartum concentrations of ammonia could be a result of the accumulation of triacylglycerides in the liver (Yung et al., 1996). Ammonia is toxic to animal tissues and therefore is rapidly converted into urea in the liver (Sinclair et al., 2000b). Circulating ammonia is quickly converted in the liver into urea. Urea is then released back into the blood stream and either excreted proportionally through urination via the kidney or recycled, mostly for use in the rumen (Jonker et al., 1998). Another source of blood urea is the metabolism of amino acids originated from RUP to ammonia in the rumen, which is then either converted to microbial proteins or absorbed into the blood stream (Butler, 1998).

Urea is the metabolic end product of protein catabolism in the body and is easily measured by the nitrogen content (Butler, 1998). As it circulates through the blood, its concentrations equilibrate into all body fluids due to its small neutral molecular size. Thus, a high correlation ($r^2 = 0.84$) between urea concentrations in milk (MUN), blood (BUN) or plasma (PUN) is established (Oltner and Wiktorsson, 1983). Thus, milk urea has been widely used to monitor protein intake and reproductive performance in dairy cows since it could be easily measured (Ropstad and Refsdal, 1987) and is more practical and less expensive than PUN analysis (Baker et al., 1995).

High dietary protein results in high concentrations of urea in plasma and milk of dairy cows, and their dosage helps measure efficiency of protein utilization (Jonker et al., 1998; Kenny et al., 2002). Plasma urea concentrations were significantly higher in cows fed on high nitrogen pasture than those fed moderate levels (Rhoads et al., 2006; Ordonez et al., 2007). Increasing protein level in diets from 12.7% to 23.2% has significantly increased the mean concentration of urea and ammonia in milk and plasma (Kenny et al., 2001).

1.4.2. Excess dietary protein or high concentrations of urea and reproduction

1.4.2.1. Excess dietary protein and reproduction

Negative impact of an excess of dietary protein on reproductive performance of dairy cows has been already extensively documented (Jordan and Swanson, 1979; Ferguson et al., 1988; Elrod and Butler, 1993). The negative association between dietary protein intake and reproduction has been recently the topic of a meta-analysis (Lean et al., 2012). When it exists, the reduction of reproduction parameters ranged between 5-28 and 6-25 points of percentage for pregnancy and CR, respectively.

However, these differences were not always significant. Indeed, some results showed that these differences trend to be more significant if trial were restricted to older cows only (Kaim et al., 1983; Bruckental et al., 2010), suggesting that a combined effect of protein level and age could be more detrimental. Similarly, effect of excess dietary protein on reproductive performance has been shown to be dependent upon other factors such as age, breed, management, other feed components and health disorders (Carroll et al., 1988).

Increased CP (12.7 to 19.3%) has also been associated with fewer services per conception (1.47 vs. 2.47) and fewer days open (69 vs. 106) than cows fed high CP diets. (Jordan and Swanson, 1979). However, another study demonstrated that high-protein diets tended to increase days open only if cows had other major health problems such as retained placenta and metritis (Barton et al., 1996).

1.4.2.2. High urea and reproduction

Extensive literature analysis reports the association between high urea and ammonia and deteriorated reproduction performance. A recent meta-analysis on the relationship between high milk or blood urea and pregnancy or conception focused on defining the appropriate urea threshold associated with this issue and qualifying RR (Annexes 1). It included 61 different models from 21 papers. The meta-analysis showed 43% lower odds of pregnancy or conception (OR [95%CI] = 0.57 [0.45-0.73]) in cases where urea ≥ 7.0 m*M* in the blood (PUN = 19.3 mg/dl) or where urea ≥ 420 mg/L in milk compared to cases where the urea values were lower (Table 10). This threshold is the most suitable with regards to pregnancy or conception success, even if a threshold of 6.5 m*M* cannot be excluded with certainty. These thresholds are (i) in accordance with those previously proposed in a review (6.9-7.2 m*M* of urea; BUN) or MUN = 19-20 mg/dl) (Butler, 1998) but (ii) above the milk urea concentrations that are usually recommended in France (250-350 mg/L) or the objective (milk urea = 250-350 mg/L or MUN = 11.5-14.0 mg/dl) proposed in a recent review (Melendez et al., 2003). Amazingly, the present meta-analysis did not allow to distinguish pregnancy, conception or conception at AI1, from both the RR or OR point of view and the threshold point of view, although theses 3 indicators represent different biological items. Moreover, the meta-analysis also confirmed the equivalency of blood or milk urea as indicator of nitrogen excess, in agreement with the literature (Butler et al., 1996). Lastly, the results also highlighted the possibility of a stronger association between high urea concentrations and pregnancy or conception failure when high nitrogen exposure occurs before AI compared to after AI, but this possibility needs to be further studied.

Table 10. Risk of pregnancy associated with blood urea values obtained via meta-analysis (Annexes 1)

Urea blood values	OR of pregnancy	95% CI
≤ 4.9 m*M* [≤ 294 mg/L]	0.95	0.89 - 1.02
5-5.9 m*M* [300-354 mg/L]	0.96	0.90 - 1.02
6-6.9 m*M* [360-414 mg/L]	Reference	-
7-7.9 m*M* [420-474 mg/L]	0.57	0.45 - 0.73
≥ 8 m*M* [≥ 480 mg/L]	0.45	0.27 - 0.73

The study suggests a stronger association between high urea and pregnancy or conception risk when the exposure occurs before AI compared to after AI. This result is in accordance with the literature (Hammon et al., 2000, Rhoads et al., 2006) despite inconsistency of the results. Ammonia was suggested to have its deleterious effect before ovulation, whereas urea probably exerts its effect during cleavage and blastocyst formation of the fertilized embryo (Jorritsma et al., 2003).

Importantly, and in accordance to the experimental design of the meta-regression, these results are valid for a change in urea around AI, and may not be extrapolated to situations with continuous high urea exposure of cows. Dairy cows were recognized to be able to adapt to high urea concentration, without any deterioration of the reproductive performance, provided that the urea concentration remains constant at high values and provided there is a wash-out period before AI (Westwood et al., 1998b). Cows could tolerate high nitrogen intake through a process of adaptation that may take less than 10 days without any significant effects on their fertility (Dawuda et al., 2002). This could explain that no effect of excess dietary nitrogen was observed on the reproductive performance of dairy cows in several studies (Kenny et al., 2001; Cottrill et al., 2002; Dawuda et al., 2002; Ordonez et al., 2007).

Other reproduction parameters are also influenced by high nitrogen or urea exposure. MUN was genetically correlated with interval from calving to first service, 56-d non-return rate and 90-d non-return rate (0.29, -0.1 and 0.12), respectively (König et al., 2008). Days to first service were 128 days in cows with MUN concentration of 20 mg/dl versus 80 days ($P < 0.05$) in cows with MUN between 16 and 20 mg/dl (MUN = milk urea × 2.8) (Gustafsson and Carlsson, 1993). Such delay has been confirmed more recently in multiparous cows (Wathes et al., 2007a): cows with concentrations > 7.5 m*M* (Approx. 20 mg/dL) needed 7 weeks longer to conceive than cows with concentrations < 4.5 m*M* (Approx. 12.6 mg/dL).

1.4.2.3. Underlying mechanisms

The link between high diet nitrogen or high blood/milk urea and the reproductive performance rely on several additive mechanisms.

First, high concentrations of urea and ammonia may change hormonal profiles of cows and affect oocyte development. High concentrations of urea and ammonia reduce LH binding to ovarian receptors, resulting in decreased ovulation rates and lower serum progesterone concentrations and consequently a delayed cyclicality (Jordan et al., 1983). Increased urea concentration has been associated with increased incidence of ovarian cysts (Ropstad and Refsdal, 1987). Reduced progesterone levels associated with high protein diets is also reported (Folman et al., 1981; Sonderman and Larson, 1989). The progesterone concentration was 30% lower at day 14 of the estrous cycle in cows fed high dietary protein compared to cows with low-protein diet (19.3 vs. 12.7%) group (Jordan and Swanson, 1979). Embryos were more likely to fertilize if they were collected from cows fed a lower level than those fed a higher level of RDP (Blanchard et al., 1990). Furthermore, excess of RDP resulted in death of embryos in 7/16 heifers on day 20 post-breeding (Elrod and Butler, 1993). Oocyte cleavage rates were reduced (61.7%) for the high PUN group compared to those of the control group (72.5%), with subsequent effects on oocyte development to the blastocyst stage, fertilization and blastocyst quality (Leroy et al., 2005). This is in accordance with lower cleavage rates of oocytes collected from cows fed high ammonia-generating diets reported by (Sinclair et al., 2000b). Recent in vivo study demonstrated that embryos collected from lactating cows having high PUN concentrations were less likely to establish pregnancy after transfer to recipients than embryos collected from cows with moderate PUN (Rhoads et al., 2006).

On the contrary, some studies reported no association between nitrogen levels in diet and the follicular (Laven et al., 2004) or the embryonic (Dawuda et al., 2002) development. The relative importance of nitrogen excess and NEB in these above mentioned changes remain unclear. The nitrogen excesses are suspected to act indirectly by altering energy status (Jordan et al., 1983; Howard et al., 1987). A possible interaction between excess protein intake and the severity of NEB during early lactation is highly suspected to be involved (Oldham, 1984). An increased intake of protein reduces the availability of energy and could intensify NEB as it requires more energy to metabolize excess protein. Every gram of excess nitrogen from overfeeding CP can increase the energy requirements by 13.3 kcal of digestible energy and may exuberate and prolong the NEB after calving (Butler, 1998). A carryover effect of energy status could exacerbate the sensitivity of reproductive organs to urea and ammonia (Butler, 2005). The energy status of the cows may also contribute to the severity of high dietary protein effect on reproductive performance (Carroll et al., 1994)

Second, the direct toxicity of ammonia or urea for oocyte and embryonic development may be involved (Jordan et al., 1983; Elrod et al., 1993; Garcia-Bojalil et al., 1994). The toxic effect of their high blood concentrations on reproductive organs has also been reported (McEvoy et al., 1997). Urea concentration in follicular fluid was highly correlated to the PUN concentrations in dairy cows (Hammon et al., 2005). The concentrations of both ammonia and urea equilibrate within reproductive fluids and urea concentration was positively associated with blood ammonia and urea concentrations when animals were fed high CP diets (Elrod and Butler, 1993). Urea and ammonia concentrations in the ovarian follicular fluids were highly correlated (r^2 = 0.86) to their concentrations in cows blood

(Hammon et al., 2005), suggesting that the oocyte within the developing follicle is susceptible to damage by high PUN concentrations. Such alterations were also observed in uterine secretions in cows fed on high protein diets (Jordan et al., 1983).

High ammonia concentration in the follicular fluid was associated with reduced cleavage rates of the oocytes (Sinclair et al., 2000a), impaired growth of granulosa cells and impaired ability to support the in vitro maturation of oocytes (Hammon et al., 2000). Oocytes matured in the presence of urea differed from those matured in a normal environment (De Wit et al., 2001). Furthermore, the percentage of embryonic development has been significantly decreased (18.2%) in the presence of urea compared to the control group (23.9%).

Third, high concentrations of urea are associated with a decreased uterine pH (6.85 vs. 7.13) in cows fed excess dietary protein than in cows fed normal levels on day 7 post-breeding, respectively (Elrod et al., 1993). Such reduction in the uterine pH is deleterious to the embryonic survival. Results in vitro showed that no embryo developed to blastocyst if exposed to media with pH values < 6.6 (Ocon and Hansen, 2003). In addition, an alteration in the ionic composition (K, Mg, Zn and P) of the uterine fluid (Jordan et al., 1983) has been observed, resulting in abnormal disturbances in follicular development and a suboptimal uterine environment for embryonic growth which could compromise the reproductive process (Elrod et al., 1993). This result was confirmed by the production of abnormal embryos that could have less ability to survive within uterine fluids having similar changes in ion concentrations (Wiebold, 1988).

Lastly, the direct negative association or nitrogen excess on immunity has been reported recently (Raboisson et al., 2014a), suggesting a potential higher risk for immune-related disorders in cows exposed to high nitrogen levels.

1.4.3. Low levels of dietary nitrogen

Low blood urea results from low dietary CP levels or diets containing inadequate protein for optimal microbial protein synthesis. The majority of the investigations focused on the effect of high levels of urea on reproduction, but few dialed with the relationship between low concentrations of urea and the reproductive performance. This question refers to the increasing number of observations of herds with a voluntary very low milk urea in the field. In the recent meta-analysis (Annexes 1), a tendency toward better pregnancy or conception in the reference class (urea = 6-7 m*M* or 360-420 mg/L) compared to lower classes, even if not significant, suggests a favorable effect of moderate urea concentration and shows that there is no evidence to support lowering urea value to improve pregnancy or conception.

A reduced pregnancy rate ($P < 0.05$) amongst cows with either high (greater than 7.0 mM) or low (less than 4.0 mM) milk urea concentrations was recorded (25 and 31.4 , respectively) compared with cows with intermediate concentrations (51.4%) (Pehrson et al., 1992). Similarly, a significant curvilinear relationship between the milk urea and the interval between calving and conception (Gustafsson and Carlsson, 1993), with the shortest interval occurring for values between 4.5 and 5.0 mM. A tendency towards longer intervals from calving to conception (79.3 vs. 72.7) and more services per conception (1.7 vs. 1.5) required for dairy cows with low milk urea relative to those with higher (≥ 2.5 mM) concentrations was described (Miettinen, 1991). During two consecutive periods of housing, cows with low milk urea (< 2.9 and < 2.5 mM) had significantly ($P < 0.05$) greater intervals from calving to first service (98 and 99) than cows with intermediate concentrations (82 and 82) (Carlsson and Pehrson, 1993). They reported same extension for calving to last service, and calving to conception.

Low milk/blood urea may reflect inadequate dietary intake of other nutrients and poor reproductive performance may result from a more severe NEB, a reduced nitrogen balance or from mineral or other dietary deficiencies (Westwood et al., 1998).

1.5. Discussion and conclusion

A clear evidence has been afforded about the association between different peripartum disorders and the reproductive performance of the dairy cow. Mastitis, both clinical and subclinical, have been negatively associated with reproduction failure and the timing of the disease has been shown to be determinant with a more deleterious effect when it occurs after AI. However, how the dynamics of the inflammation is associated with conception change is still poorly documented. The evolution of the inflammation around AI could play an important role in the magnitude of the association with the reproductive failure. In addition, the majority of these studies investigated the association between mastitis and reproduction without considering other diseases that occur simultaneously. Peripartum metabolic disorders, such as subclinical ketosis, were also reported to be negatively associated with reproduction. Although one particular disorder by itself may not have a large effect on reproduction, multiple disorders can exacerbate the individual effects and have additive detrimental effects on reproduction (Ahmadzadeh et al., 2010).

To sum up, in spite evidence on associations between the above mentioned disorders are available, there is still challenges to be faced, in particular regarding the quantification of the strength, the conditions in which these associations remain valid, and the presence of multiple interactions between these associations.

There are a lack of reports on the complex interactions amongst these events. Genetic correlations between milk production traits, reproductive performance and diseases (including mastitis, lameness, milk fever, ketosis and tetany) were all unfavorable: they ranged from 0.07 to 0.37 for milk production and diseases, and from 0.06 to 0.41 for diseases and poor fertility (Kadarmideen et al., 2000). Previous works (Loeffler et al., 1999a; Fourichon et al., 2000; Maizon et al., 2004) highlighted that diseases, other than mastitis, including ketosis, strengthened the negative effect on the reproductive performance of dairy cows. Cows with mastitis associated with other peripartal diseases had greater ($P < 0.05$) number of days non-pregnant compared with cows with mastitis only or with other diseases only (155, 140 and 97 day) and greater number of services per conception (2.8, 2.1 and 1.9), respectively (Ahmadzadeh et al., 2009). It showed that the detrimental effects of CM on reproduction are more evident when cows experience both CM and other diseases. Increased incidence of CM in high-producing cows could be due to the impairment of udder defense mechanisms linked to hyperketonemia (Suriyasathaporn et al., 2000). Indeed, 28.6% of the cows with prepartum ketosis subsequently developed CM compared with 8.7% of the cows without prepartum ketosis (Leslie et al., 2000). Cows that had been diagnosed with ketosis were almost twice as likely to have a mastitis diagnosed in the first 35 d of lactation (Berge and Vertenten, 2014). A similar association was reported by (Oltenacu and Ekesbo, 1994). These studies showed that peripartum diseases could generally be associated, but the direct association and the quantitative effect of these interactions remain yet uninvestigated. Although a limited number of studies showed a direct link between udder inflammation and SCK, the magnitude of the interaction on the reproduction performance has never been studied.

Ketone bodies represent the most reliable indicators of SCK. However, their use to evaluate the conception success, which need a large number of data, sounds of limited value since it is difficult to measure them at the herd level on a large scale. An alternative has been proposed through the fat and protein contents of the milk, which is still rarely used because of the moderate sensitivity and specificity compared to BHBA and NEFA concentrations. However, it sounds of interest to use these contents as a proxy to define SCK status at the herd level through a large database such as that provided by the milk control program (MCP) in France. The need of accurate indicator of SCK available for a high number of cows for a long period would help in better understanding the related associations.

Urea is the final product of the protein metabolism and its concentration in blood and milk are very well correlated (Westwood et al., 1998). It was widely used to measure the efficiency of protein adjustments in rations and an excess in crude protein in the diet has been widely studied. It was often negatively associated with reproductive performance. However, a remarkable variation of urea values amongst studies that might be associated with conception failure could be highlighted. A few reviews (Westwood et al., 1998; Melendez et al., 2003) tried to summarize these thresholds and reported a wide range of milk or blood urea values reported to be associated with a decreased fertility. However, these reviews never presented a quantitative demonstration of the urea threshold and were basically based on the authors' experience. Thus, it will be of a high interest to define a limit threshold in order to improve efficiency of the protein supplementation.

In addition, only few reports investigated the association between a nitrogen deficiency and reproduction. Even if a negative association between low urea levels (in blood or milk) has been found (Miettinen, 1991; Pehrson et al., 1992), these trials were done on a very small number of cows. The dynamics of urea, whatever changes are increasing or decreasing, may be key factor to study its relationship with conception: cows were shown to be able to adapt to high urea levels if they are given a sufficient time. For instance, evidence was found about a negative effect of short-term urea change that could be similarly or even more deleterious on reproduction than constantly high urea levels. In fact, the capacity of the animals to adapt to high plasma urea nitrogen was reported by (Dawuda et al., 2002), where feeding high protein diet starting 10 days before AI had no effect on the quantity and quality of embryos, whereas the same diet starting just prior ovulation reduced significantly the number of embryos collected. This was confirmed by (Laven et al., 2004) who reported that no variation in follicular development or embryos growth was found when cows were fed supplemented urea rations for 10 weeks compared to cows fed rations without supplemented urea.

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Synthèse de la problématique

La reproduction est un déterminant majeur de la rentabilité de l'élevage bovin. Les résultats de reproduction connaissent une dégradation continue depuis plusieurs décennies en France et à l'étranger. Plusieurs facteurs sont identifiés comme contribuant à cette dégradation, parmi lesquels la sélection génétique et l'augmentation de la production de lait, les désordres alimentaires et les troubles sanitaires du *peripartum*. Si une littérature abondante est disponible sur ces différentes relations, d'une part certaines relations bilatérales restent mal documentées et en particulier non précisément quantifiées et d'autre part les modalités selon lesquelles ces diverses entités interagissent sont peu à pas décrites.

Le lien entre la reproduction et la mammite, clinique ou subclinique, a été clairement établi et l'incidence de mammite après l'IA est souvent démontrée comme plus déterminante qu'avant l'IA (chapitre 1 ; section 1.2). L'impact d'une mammite sur la conception semble d'autant plus élevé que la mammite est sévère et l'augmentation des cellules élevée. Il apparaît intéressant de réévaluer cette association en focalisant sur la dynamique de l'infection et des cellules somatiques, et donc l'évolution de l'inflammation autour de l'IA, pour identifier comment la fertilité est modulée selon les différentes dynamiques d'inflammation mammaire autour de l'IA.

De plus, la cétose subclinique ou hypercétonémie a été identifiée comme négativement associée à la fertilité, avec un taux de conception significativement réduit lors d'hypercétonémie (section 1.3). Les liens directs du déficit énergétique sur les mécanismes reproductifs sont bien identifiés, mais le lien hypercétonémie–conception pourrait aussi être le résultat d'une occurrence accrue de maladies lors d'hypercétonémie, plusieurs troubles tels que les différents types de métrites ou la rétention placentaire étant observés de manière plus fréquente lors d'hypercétonémie. Etonnement, le lien entre hypercétonémie et conception est rarement quantifié, justifiant tout d'abord de réaliser cette quantification. De plus, la cétose subclinique a été associée, dans quelques essais, à un risque accru de mammites subcliniques. Ceci conduit donc à s'intéresser aux liens deux à deux et aux interactions entre la cétose subclinique et les mammites comme facteurs de risque de dégradation de la fertilité. Le gold-standard utilisé pour définir la cétose subclinique est la concentration sanguine ou dans le lait des BHBA. Les AGNE du sang sont aussi largement utilisés. Malheureusement, la mesure de ces indicateurs n'est pas disponible à grande échelle dans les conditions d'élevage. Cette limite est potentiellement à l'origine du manque de description du lien entre cétose subclinique et troubles de la reproduction et de la santé de la mamelle. Une alternative repose sur l'utilisation des taux butyreux et protéique du lait comme critère diagnostique de cétose subclinique. Si ce proxy ne représente que grossièrement la cétose subclinique, la méthode retenue rend possible l'évaluation des relations tripartites entre conception, mammites et cétose subclinique, tel que proposé dans la première partie expérimentale.

Parallèlement aux liens entre conception, mammites et reproduction, une littérature abondante décrit les relations entre l'équilibre protéique et azoté des rations et la reproduction (chapitre1, section 1.4). L'équilibre azoté des rations est un challenge alimentaire majeur en peripartum. Une supplémentation en protéines est une pratique souvent adoptée par les éleveurs pour augmenter la production laitière mais elle est souvent rapportée associée négativement avec la fertilité. L'urée constitue le produit final du métabolisme protéique et ses concentrations en plasma et en lait sont très bien corrélées : les deux ont été largement utilisées pour mesurer l'efficacité de l'alimentation protéique. Un niveau d'urée élevé est consensuellement associé avec une diminution de la fertilité mais les seuils d'urée associés sont pas clairement explicités, justifiant de re-quantifier ces éléments. L'abondance de la littérature sur ce thème conduit à recourir à la méthode de la méta-analyse. A l'opposée, peu d'intérêt a été porté aux urées du lait ou du sang basses et à leur lien avec la reproduction. Les rares données disponibles suggèrent une baisse limitée de la fertilité lors d'urée basse. Pour investiguer ces relations, il apparaît opportun d'une part d'inclure le statut énergétique aux côtés du statut protéique des animaux, ces deux pouvant interagir, et d'autre part, de considérer à la fois des valeurs basses d'urées mais aussi des baisses d'urée du lait, dans une logique dynamique. En effet, une grande capacité d'adaptation des bovins laitiers aux valeurs d'urée élevées et stables a été rapportée, suggérant une possibilité de comportement similaire pour des urées du lait basses. Les mesures de l'urée de lait sont effectuées en moyenne sur 58% des contrôles laitiers mensuels en France. Ainsi, cette grande base de données paraît adaptée pour évaluer le lien possible entre l'urée faible du lait et la réussite à l'IA.

En bilan, l'objectif de ce travail est d'abord de réévaluer l'association dynamique entre l'inflammation mammaire et la fertilité via les variations temporelles de CCS autour de l'IA en tenant compte de la présence ou l'absence de facteurs de risque potentiellement communs à ces entités pathologiques telle que la cétose subclinique. Un deuxième objectif est de ré-analyser la relation entre les déséquilibres azotés et la fertilité chez la vache laitière (i) par explorer les associations entre la fertilité et le déficit azoté évalué via les valeurs basses d'urée ; et (ii) par redéfinir le seuil néfaste d'urée en cas d'excès protéique.

2. Chapitre 2. Production, fertilité et santé mammaire des vaches laitières françaises : étude descriptive

Résumé :

L'objectif de cette partie est de caractériser à travers quelques statistiques descriptives l'ensemble des données du contrôle laitier et d'IA qui seront utilisées dans les études suivantes. Le Contrôle Laitier est une entreprise au statut associatif au service des Chambres d'Agriculture. Il regroupe 74 organisations locales directement gérées par des éleveurs élus par l'ensemble des adhérents. Ces organismes sont structurés au niveau national par une Fédération : France Conseil Elevage (http://www.france-conseil-elevage.fr/). France Génétique Elevage (http://www.france-genetique-elevage.org/) a fourni (i) les données du contrôle laitier de cette étude qui représentent l'ensemble des informations des contrôles laitiers mensuels des troupeaux français de la période 2008-2012, et (ii) les données qui concernent les femelles inséminées pour la même période. Les enregistrements des valeurs de l'urée ont été fournis par France Conseil Elevage.

Les principaux résultats montrent que le taux de conception s'est stabilisé durant la période de cette étude (2008-2012). Cette stabilité a été associée à une production laitière constante au cours de la même période. Les vaches Prim'holstein sont les moins fertiles. Des variations régionales ont été observées. Elles peuvent être expliquées par les proportions des races dominantes dans chaque bassin laitier mais aussi par des interactions saisonnières, avec plus d'IA réalisées pendant l'hiver, période où elles ont le plus de chance de réussir, que pendant le reste de l'année. Une corrélation linéaire entre la parité et le taux de conception a été observée. Les chances de conception augmentent quand l'IA est réalisée entre 50 et 100 jours de lactation. Par ailleurs, l'analyse des intervalles entre 2 IA successives montre un taux élevé de retours en chaleur tardifs (> 35 jours). Ceci témoigne d'une mortalité embryonnaire précoce fréquente, et/ou d'un défaut de détection du retour en chaleur à 21 jours.

En préparation pour publication

Production, reproduction and udder health results of French dairy cows: an extensive description

ALBAAJ

Interpretive summary

Reproductive performance has been decreased continuously worldwide and several factors could be associated with this degradation. This work showed that CR of French dairy cows stabilized within the period 2008-2012. Conception rates varied considerably amongst the different production areas. Results suggest a high rate of early embryonic death or of heat detection failure.

FERTILITY OF FRENCH DAIRY COWS

Production, reproduction and udder health results of French dairy cows: an extensive description

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2.1. Introduction

Dairy cow fertility is a key driver of the efficiency of farm management and its relationship with dairy production is a major concern for farmers because of the related economic losses (Groenendaal et al., 2004). Several reports show a continuous decrease in reproductive performance all around the world (Royal et al., 2000; Lucy, 2001). The same situation is observed in France in all dairy breeds, especially in the Holstein breed (Barbat et al., 2010).

Genetic selection for high milk production has always been suspected as one of the reasons of deteriorated reproduction: negative genetic correlation has been evidenced between milk production and reproductive performance (Boichard et al., 1999). However, despite the common belief of an overall antagonism between MY and fertility, some evidence was recently provided that this relationship is more complex than previously thought, and other physiological environmental and management factors could be strongly implicated (LeBlanc, 2013; Bello et al., 2012).

The full understanding of the current situation requires identifying the interactions between all the above-mentioned factors to better manage the current negative trends in dairy cattle health. This section aims on the one hand to describe the evolution of reproductive status of French dairy cows and on the other hand, to discuss the principal factors associated with this status.

2.2. Materials and methods

The data included in this study contain records from herds in the Milk Control Program (MCP) in France from 2008 to 2012. They were provided by France Génétique Elevage (http://www.francegenetique-elevage.fr/). These records included the lactation number, calving date, all test-day milk results, and lactation data (length and production) for all lactations. The French MCP represented 61, 57 and 85% of the herds, cows, and milk produced, respectively. The representation of dairy cows included in the MCP varies amongst dairy production areas (DPA) and ranges from 40% to 67%. Measurements of milk urea are optional for farmers registered in the MCP and are conducted on an average of 57.7% of the test-days. The records for milk urea during the same period were provided by France Conseil Elevage (http://www.france-conseil-elevage.fr/). France Génétique Elevage also provided data on AI, including identities of the dams and sires and the dates of all AI performed on heifers and lactating cows. The database was created using the open tools phpMyAdmin and MySQL (Version 5.0.51).

AI was considered successful if the cow delivered a calf with a gestation period of 265-295 days. This duration was defined as the average period of gestation for Holstein cows ±15 days, as recommended by the French Livestock Institute. If two AI were followed by a gestation period that is included in the mentioned range, the closest one to the mean gestation period (280 days) was considered as the successful one, the other was excluded from the analysis.

Three statistical approaches were retained. First, a survival analysis was performed to investigate the relationship between MY and DIM at AI1 for cows from all years of the study, distinguishing the breed classes. The survival probability was obtained by calculating the Kaplan-Meier estimator (package survival of R), disregarding the fact that a dependence on herd might exist. For all cows, the Kaplan-Meier estimator was calculated from 40 to 200 DIM, and from 2000 to 12000 kg of MY (quantiles 1 and 99% were excluded).

Second, a logistic regression with a Poisson correction was performed using the nlme package (https://cran.r-project.org/package=nlme). A log-link model with a Poisson distribution was used to calculate RR rather than OR and to simultaneously address the lack of convergence sometimes observed in log binomial regressions (Greenland, 2004; Zou, 2004; Ospina et al., 2012). This model was applied yearly to each explanatory variable separately and Herd was kept as random variable in all models.

Third, an analysis of variance procedure followed by Tukey's (HSD) test was designed to test for a significant difference in conception success as between the different breeds of dairy cows, parity groups, DPA, and months of AI. The analysis was made on the difference between the mean CR of each group of cows in each herd.

Three seasonal periods were identified which correspond to (i) the cold season: from November to February (ii) the moderate season: March, April, September and October (iii) the hot season: from May to August.

2.3. Results

2.3.1. Overall characteristics of the data

A brief description of the content of the dataset is shown in Table 11. The number of cows include cows present at least one test-day in a farm for the given year. A cow might have 2 registered lactations in the same year. The causes of non-qualification of a given lactation include amongst others a very long interval between calving and first test-day, 2 months (or more) without test, 2 very long intervals between 2 successive test-days within the same lactation.

Table 11. Descriptive characteristics of the data set for the 5 years included in this study (2008‑2012) and a statistical summary of some variables of interest for cows used in this work

	Year				
	2008	2009	2010	2011	2012
Total number of test-days	24,011,139	22,964,178	22,306,396	22,578,035	22,487,534
Number of dairy cows	3,420,486	3,300,955	3,217,129	3,264,403	3,123,891
Number of lactations	4,660,944	4,460,886	4,339,450	4,400,609	4,211,508
Number of qualified lactations	3,537,648	3,347,378	3,271,693	3,284,745	3,101,382
1^{st} parity (%)	36.2	35.6	36.3	37.8	37.9
2^{nd} parity (%)	25.7	26.3	25.8	25.6	26.5
3^{rd} parity (%)	17.2	17.2	17.4	16.8	16.5
$>3^{rd}$ parity (%)	20.9	20.9	20.4	19.8	19.2
MY (kg)					
Mean	7612	7585	7732	7938	7856
SD	1906	1938	1962	2022	2030
Min	2418	1989	1845	1882	1876
Max	12277	12262	12365	12705	12703
Number of cows with at least one AI	3,045,079	2,984,069	2,997,266	3,012,580	3,005,256
Number of total AI	5,322,875	5,140,458	5,171,924	5,235,058	5,237,335
Conception rate (%)	41.7	41.4	41.6	40.6	41.5
Number of first AI	2,648,174	2,573,773	2,568,674	2,582,734	2,561,744
Conception rate (%)	47.1	47.6	48.5	47.5	48.7

As expected, the conception is higher for heifers compared to multiparous cows and for Montbéliarde and Normande breeds compared to Holstein (Table 12). The structure of the herds included in the datasets (milk control programs), the use of AI, and the results of conception did not show differences between years for the period studied (Tables 11 and 12).

Table 12. Average conception rates for heifers and lactating French dairy cows for the 5 years of the study.

	Year				
	2008	2009	2010	2011	2012
All breeds included					
All cows	41.7	41.4	41.6	40.6	41.5
Heifers	63.2	63.7	63.4	62.6	62.3
Lactating cows	37.6	36.8	37.0	36.1	37.3
Holstein					
All cows	40.2	39.9	40.3	39.5	41.1
Heifers	65.4	66.5	66.7	66.6	69.5
Lactating cows	35.1	34.2	34.3	33.5	34.8
Montbéliarde					
All cows	48.1	47.6	48.1	47.4	48.5
Heifers	65.0	64.9	64.9	65.7	69.7
Lactating cows	45.9	45.3	45.4	44.5	44.9
Normande					
All cows	46.4	47.1	47.3	45.2	47.2
Heifers	67.6	68.0	68.7	66.9	70.5
Lactating cows	41.7	42.0	42.1	40.1	41.7

The French territories are divided into 11 DPA according to the industrial organization of the milk collection, but they overlap approximately with the French livestock systems (Raboisson et al., 2011) as shown in Figure 2.

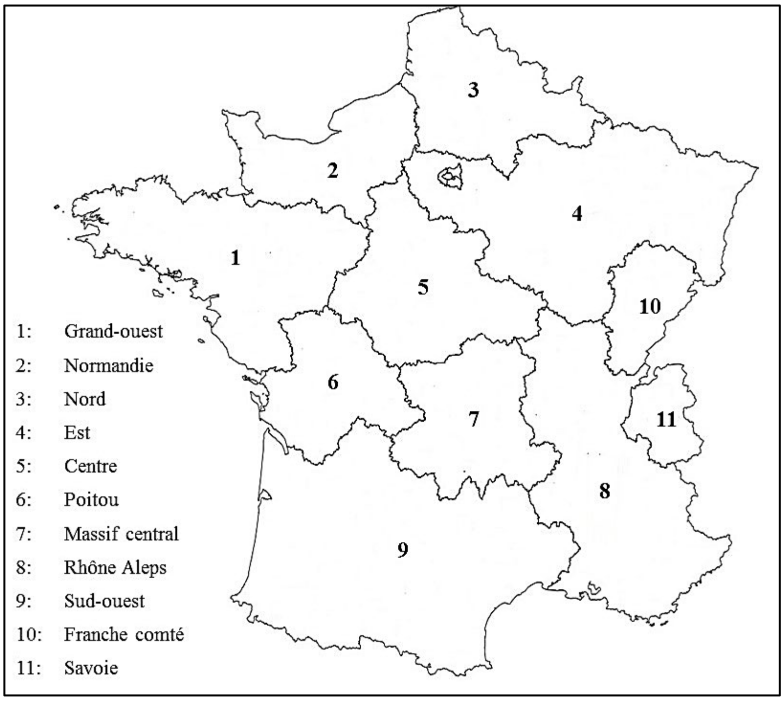

Figure 2. Definition of the dairy production areas (DPA)

The temporal pattern of milk yield, fat, protein and SCC is reported in Figures 4, 5, 6, 7 and 8. The respective spatial pattern reported in Figures 9, 10, 11, 12 and 13. Temporal changes in daily milk production are due, among others, to date of calving (Figure 3). The increase in SCC in summer is partly linked to environment temperature, as suggested by differences in SCC for the different DPA.

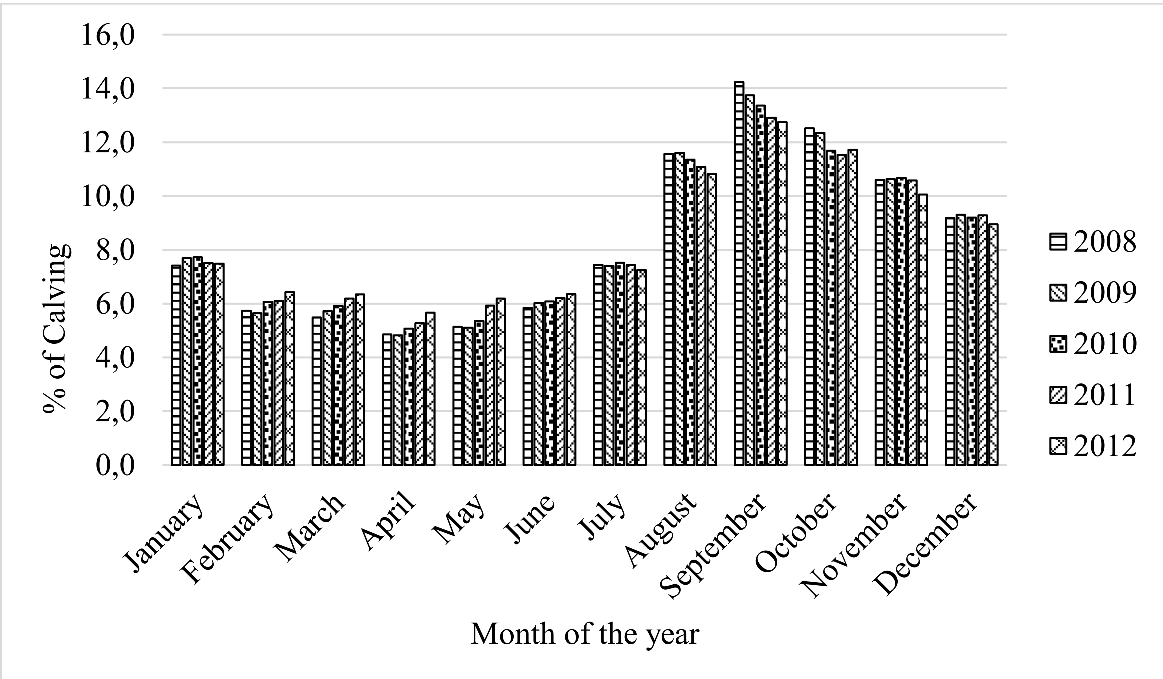

Figure 3. Yearly distribution of the within-year calving proportion in French dairy herds from 2008 to 2012

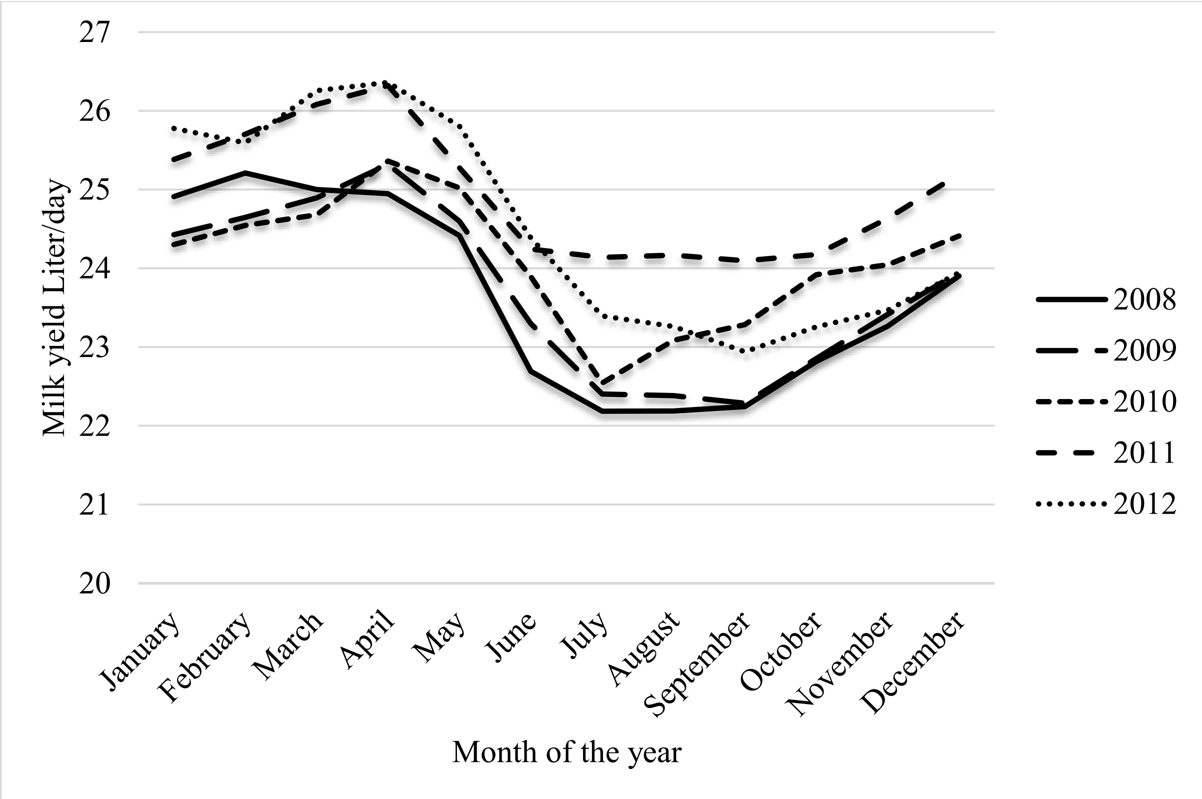

Figure 4. The monthly variation of the average daily milk yield (Liters) produced by French dairy cows within each year of the study

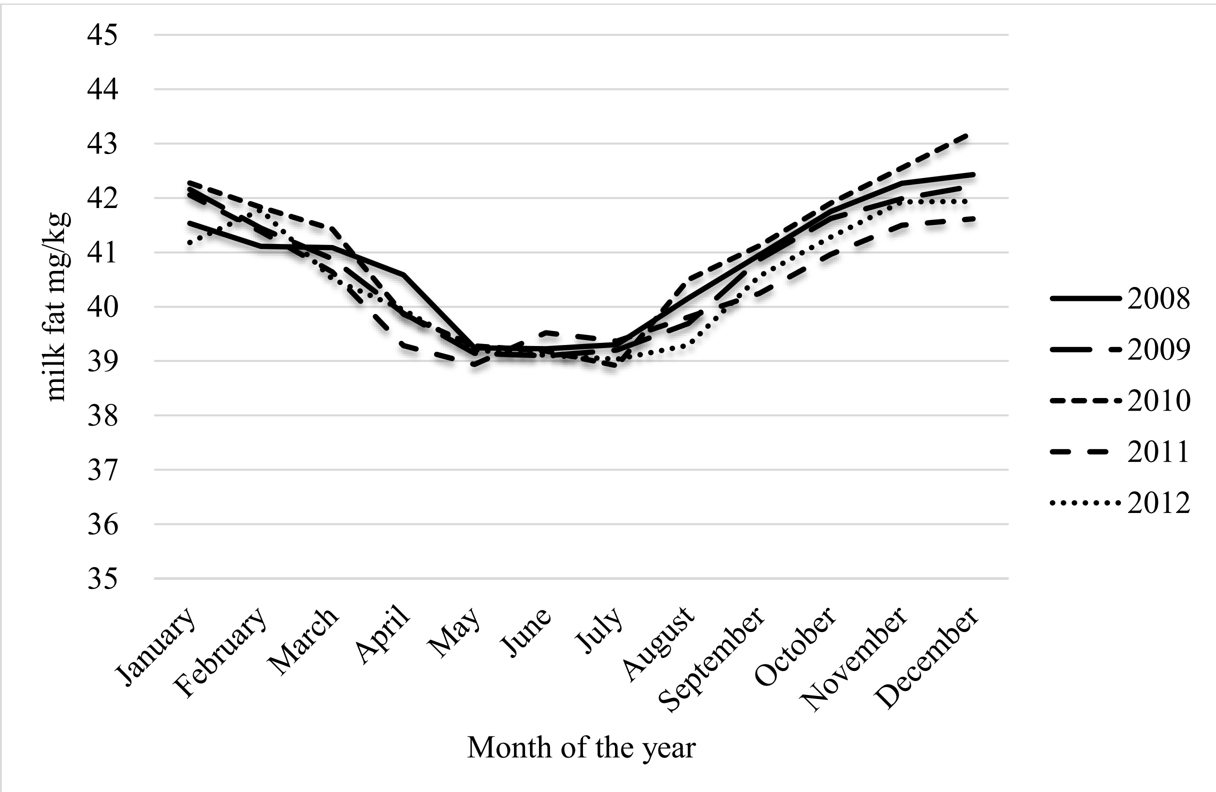

Figure 5. The monthly variation of the average daily milk fat content (mg/kg) for French dairy cows within each year of the study

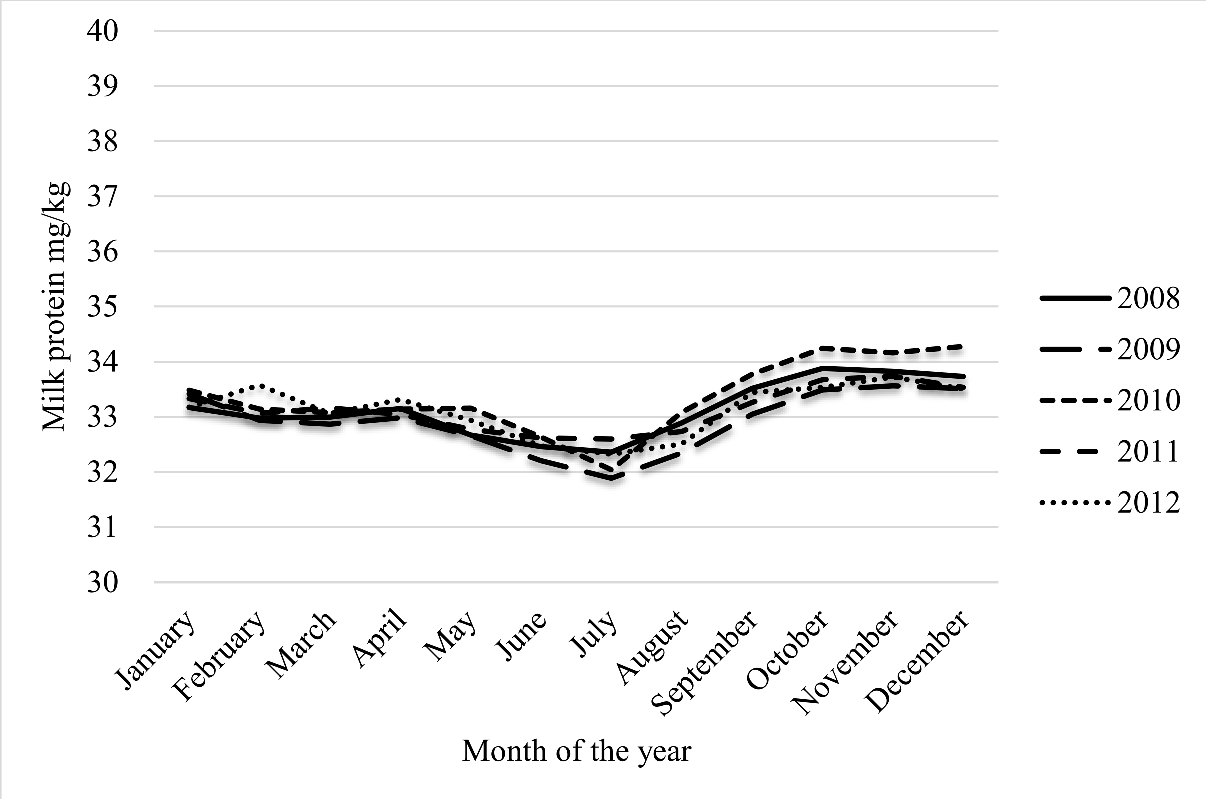

Figure 6. The monthly variation of the average daily milk protein content (mg/kg) for French dairy cows within each year of the study

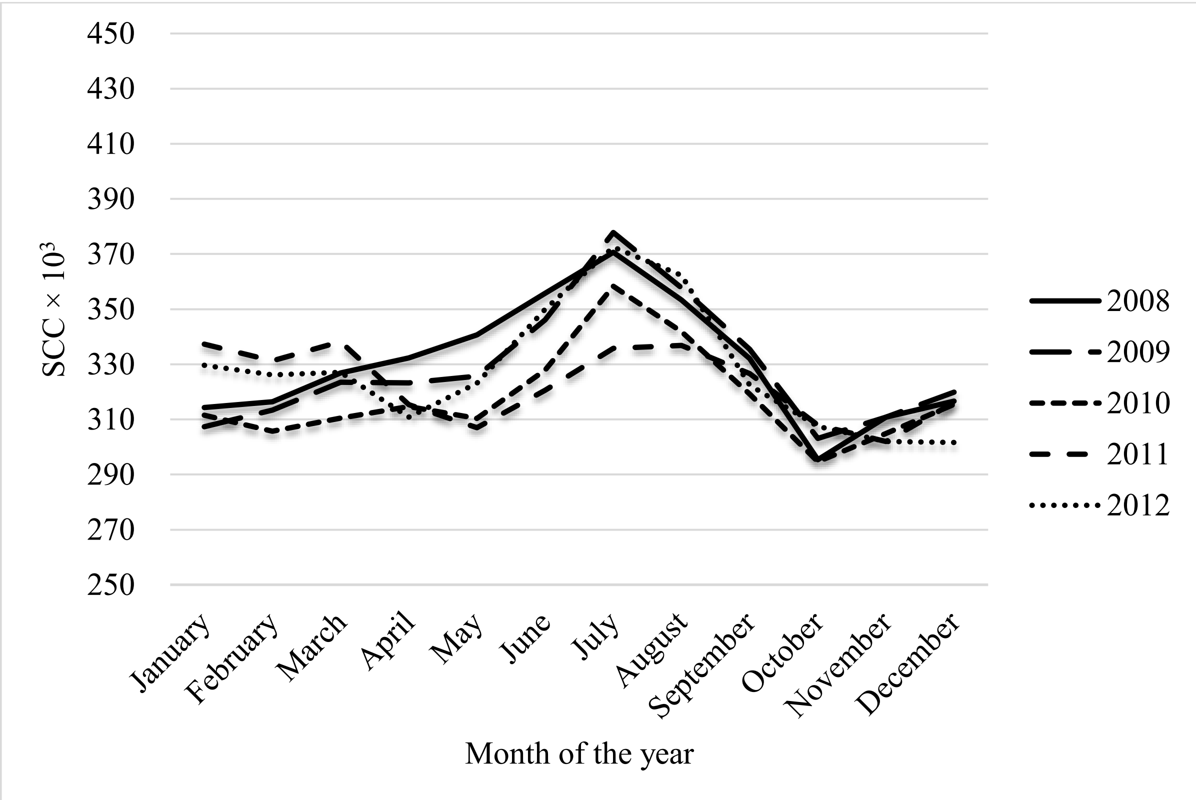

Figure 7. The monthly variation of the average SCC ($\times 10^3$) in the milk of French dairy cows within each year of the study

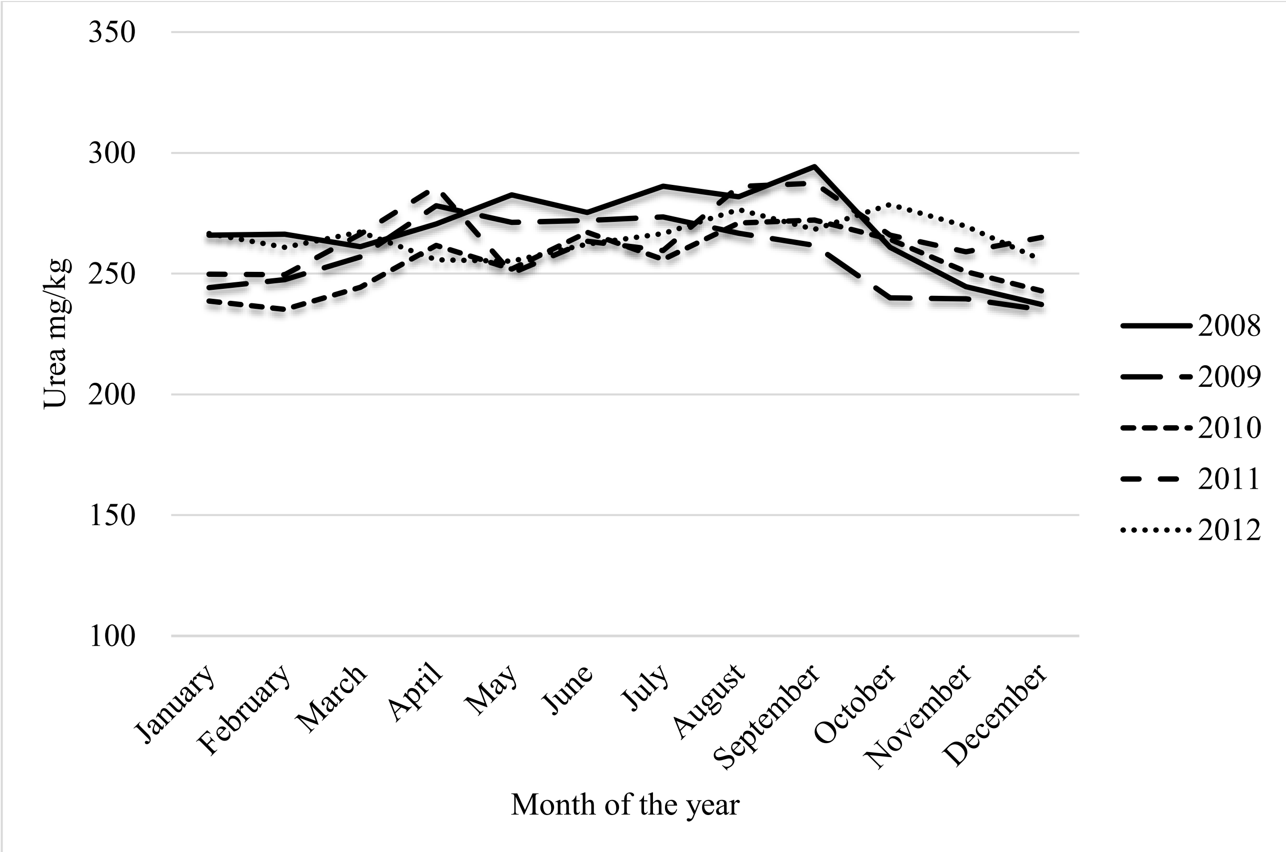

Figure 8. The monthly variation of the average urea concentration (mg/kg) in the milk of French dairy cows within each year of the study

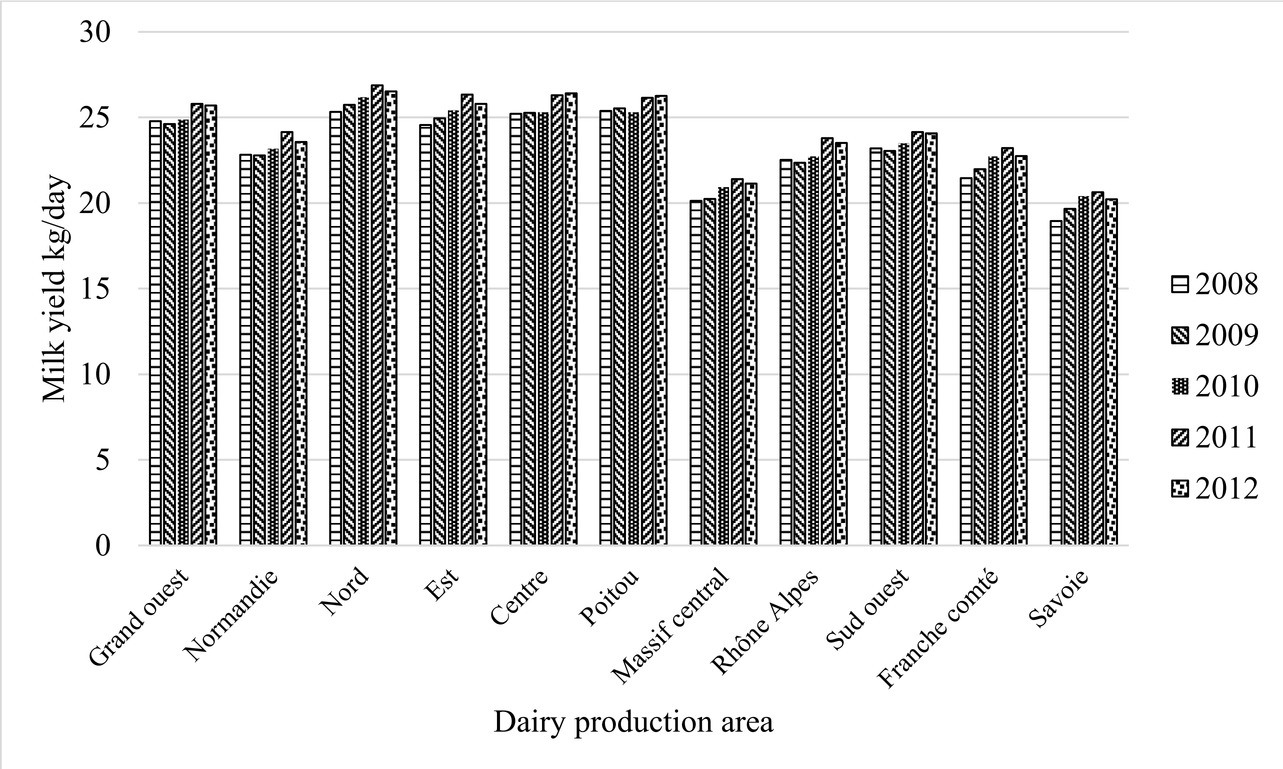

Figure 9. Variation of daily milk yield (kg) produced within each dairy production area (DPA)

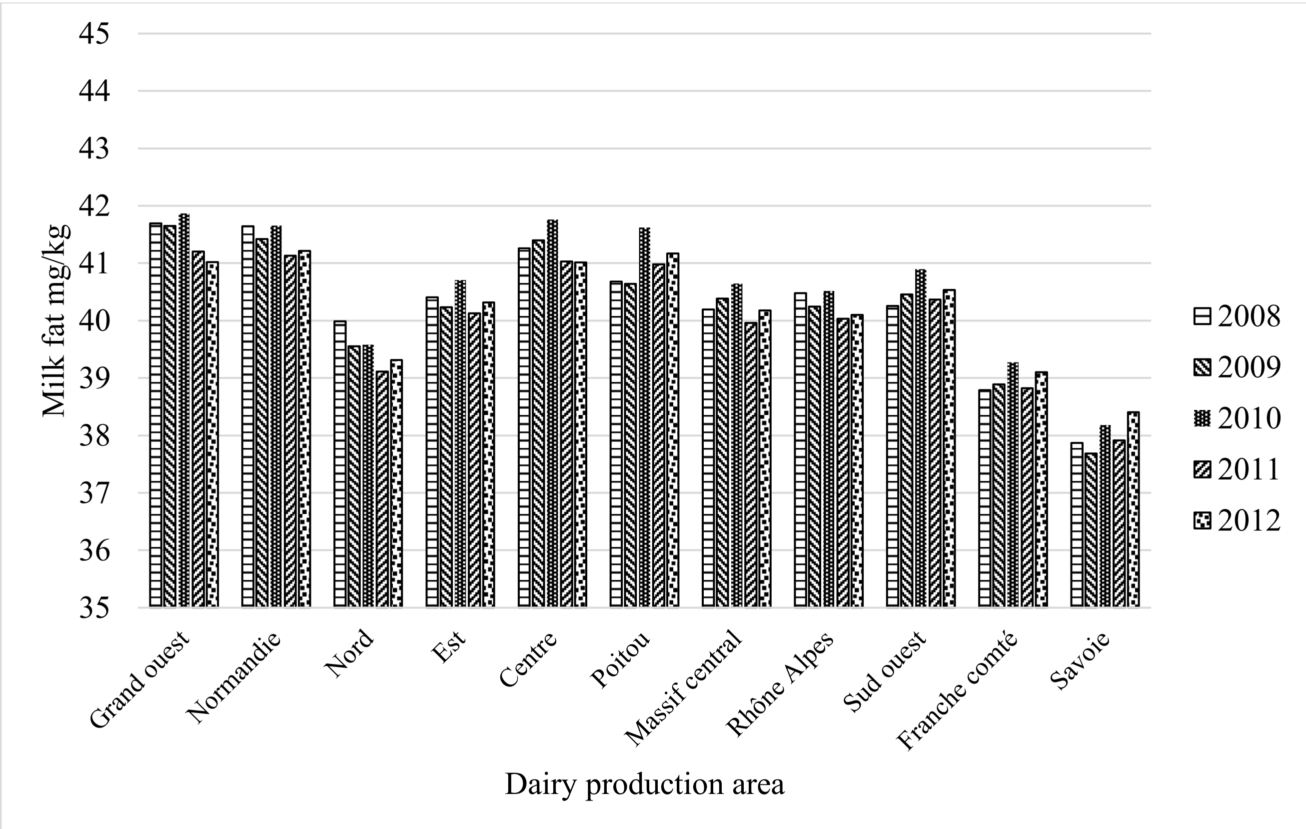

Figure 10. Variation of daily fat content (mg/kg) in milk produced within each dairy production area (DPA)

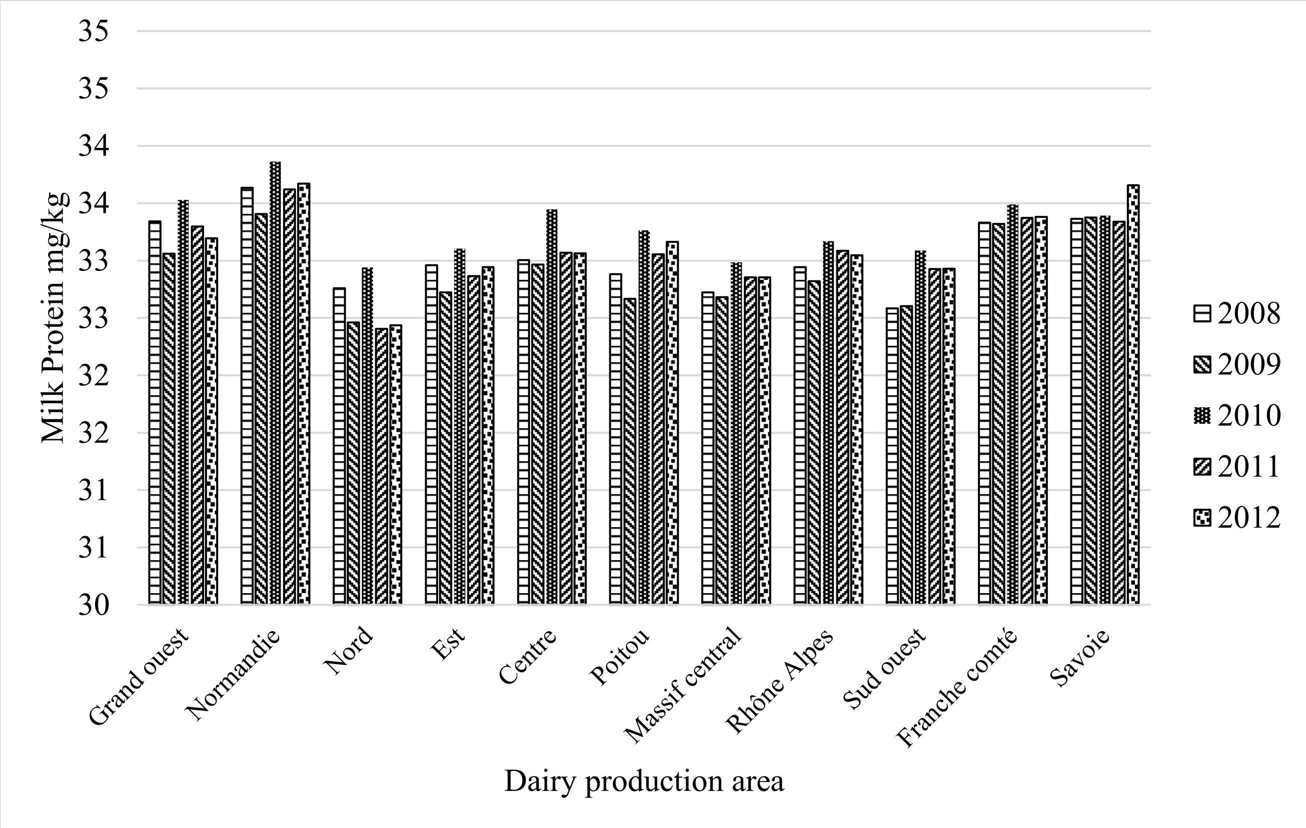

Figure 11. Variation of daily protein content (mg/kg) in milk produced within each dairy production area (DPA)

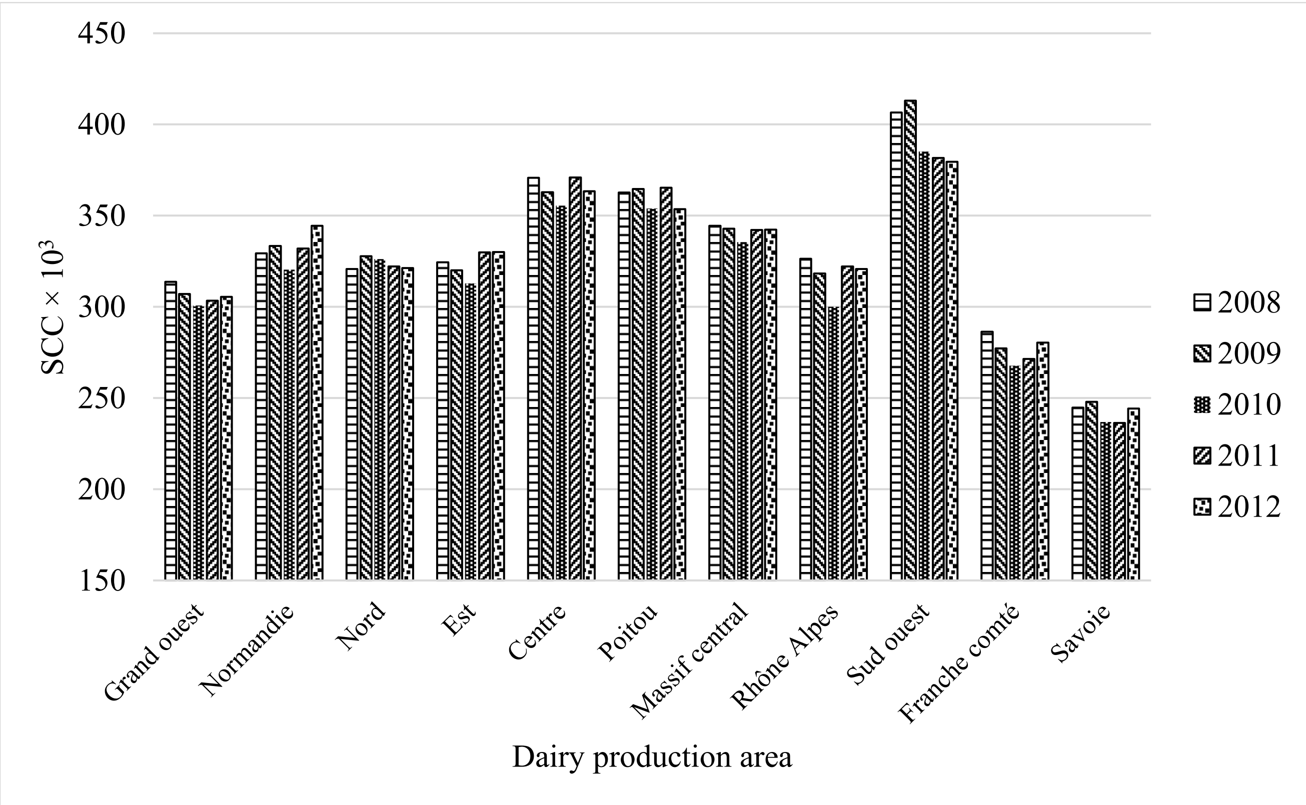

Figure 12. Variation of average SCC ($\times 10^3$) in milk produced within each dairy production area (DPA)

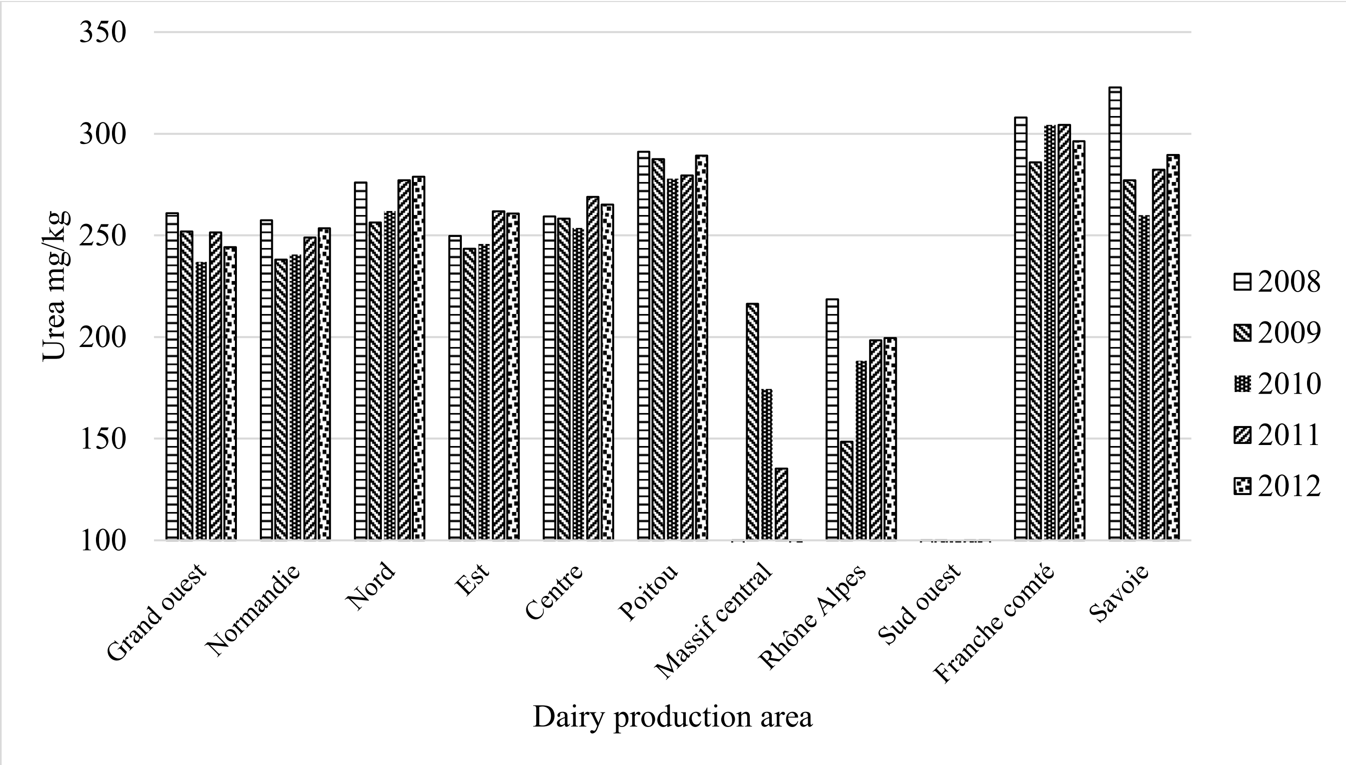

Figure 13. Variation of average urea concentration (mg/kg) in milk produced within each dairy production area (DPA)

2.3.2. Milk yield and conception

The yearly CR is almost the same for the whole period, in spite of a slight decrease when production increased (Figure 14). Mean 305-days MY (kg) produced by each breed and by parity are represented in Table 13 for the whole period of the study. Table 14 and Figure 15 highlighted the negative relationship between the production level of a given breed and its average CR. Montbéliarde and Normande were for instance more likely to conceive (RR from 1.25 to 1.31 and from 1.20 to 1.24, respectively) compared to Holstein (Table 14). Interestingly, Montbéliarde cows produced more milk than Normande ones, but have the highest CR, showing the multifactorial determinants of conception success. This suggests crossing breed, areas and milk production for the analysis.

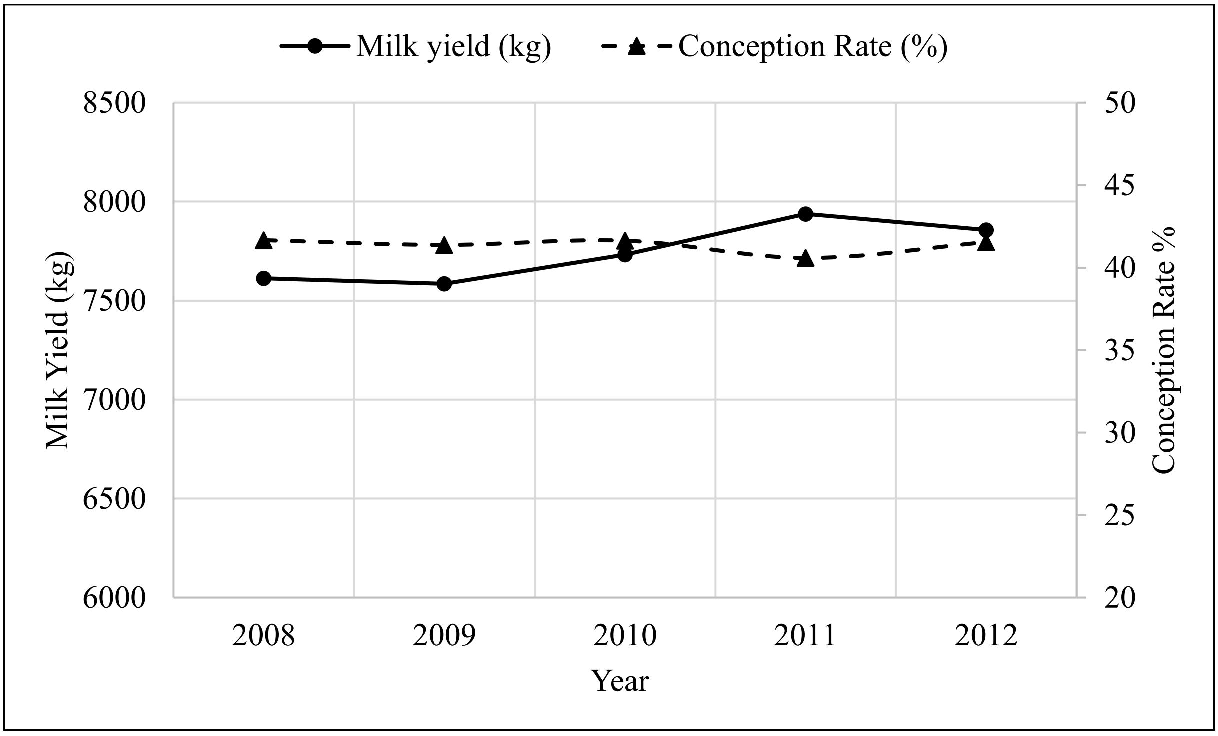

Figure 14. Mean conception rates (%) and average 305-days milk yield (kg) from French dairy cows over the five years of the study

Table 13. Mean 350-days milk yield in kg (±SD) produced by french dairy cows within the period 2008-2012

	Breed			
Parity	Holstein	Montbéliarde	Normande	Others
First	7033±1920	5610±1627	5221±1446	5399±1909
Second	7712±2371	6212±1951	5605±1762	5853±2240
Third	7860±2468	6429±2043	5845±1865	6034±2298
> third	7486±2571	6119±2160	5712±1968	5644±2276

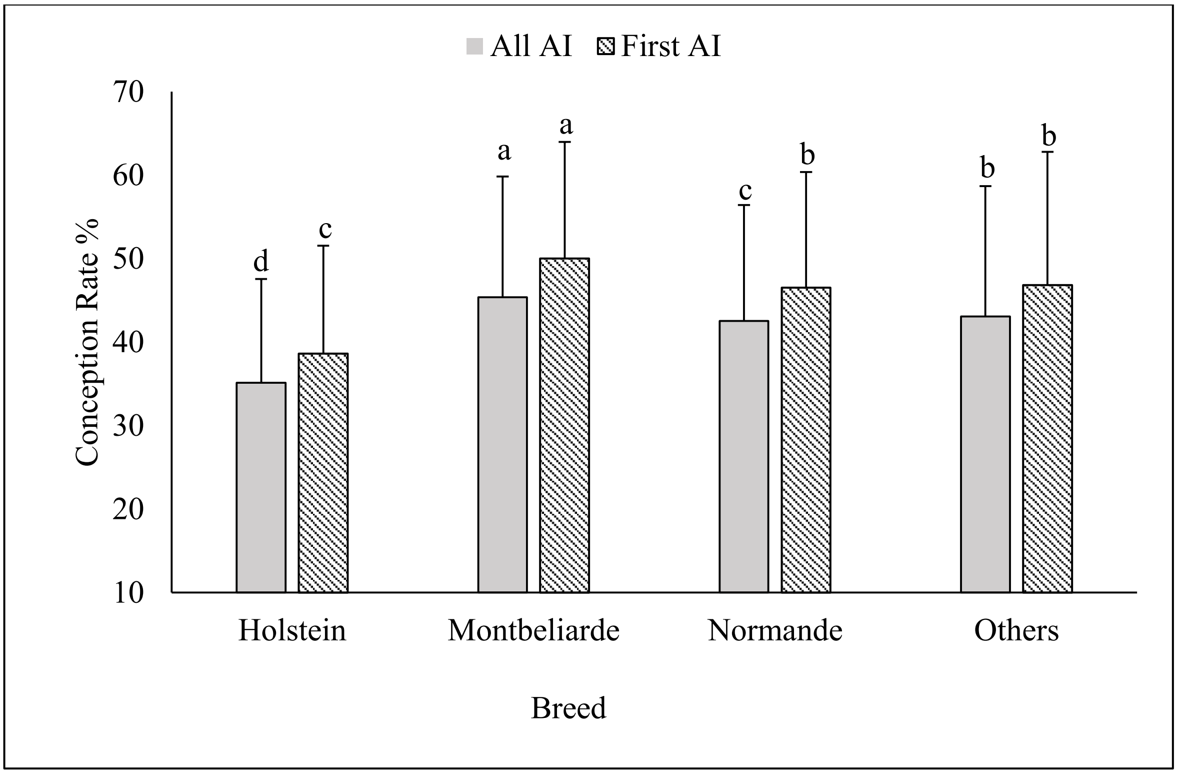

Figure 15. Conception rate (%) at all and at AI1 for the main French dairy breeds over the period 2008-2012

Table 14. Relative risks (95% CI) of conception at all AI associated with breed (compared to Holstein) within each year of the study.

Year	Breed			
	Holstein	Montbéliarde	Normande	Others
2008	Referent	1.27(1.27-1.28)***	1.20(1.19-1.21)***	1.22(1.21-1.23)***
2009	Referent	1.31(1.30-1.32)***	1.24(1.23-1.25)***	1.26(1.24-1.27)***
2010	Referent	1.31(1.29-1.32)***	1.24(1.23-1.25)***	1.25(1.23-1.26)***
2011	Referent	1.28(1.27-1.29)***	1.20(1.19-1.21)***	1.24(1.23-1.26)***
2012	Referent	1.25(1.24-1.26)***	1.20(1.19-1.21)***	1.20(1.18-1.21)***

***$P < 0.001$

2.3.3. Dairy production areas, breed and conception

Conception rate for each DPA (Figure 16) is in accordance with the main breed present and the level of production. For instance, high CR in Franche-Comté is in accordance with Montbéliarde as main breed (Figure 17). Massif-Central and Rhone-Alpes areas have a mix of breed and high CR, showing that production level and livestock system may interact to influence CR. Indeed, Holstein cows in these two DPA tend to have lower milk production compared to Holstein in other areas (Figure 18).

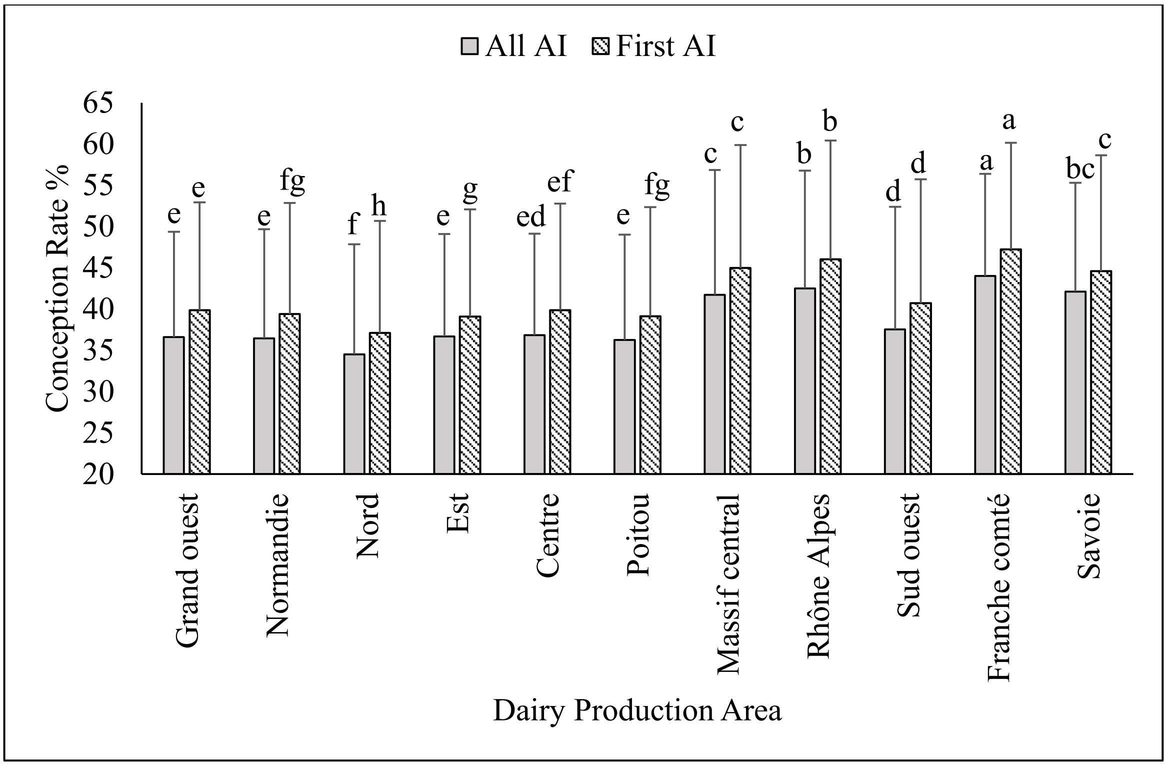

Figure 16. Conception rate (%) at all AI and at AI1 per dairy production areas (DPA)

Table 15. Relative risk (95% CI) of conception at all AI associated with dairy production areas (compared to DPA1) for cows from all breeds and for Holstein cows only for the whole period of the study.

Dairy production area	Breed	
	All breed	Holstein
Grand-Ouest (1)	Referent	Referent
Normandie (2)	0.97 (0.96-0.99)***	0.93 (0.92-0.95)***
Nord (3)	0.91 (0.90-0.92)***	0.93 (0.92-0.95)***
Est (4)	1.00 (0.99-1.02)	0.99 (0.98-1.01)
Centre (5)	1.01 (0.98-1.05)	1.03 (1.00-1.06)
Poitou (6)	0.99 (0.96-1.01)	1.01 (0.98-1.03)
Massif central (7)	1.11 (1.09-1.13)***	1.08 (1.06-1.11)***
Rhône-Alpes (8)	1.15 (1.13-1.17)***	1.05 (1.03-1.08)***
Sud-Ouest (9)	0.98 (0.96-1.00)*	0.98 (0.97-1.00)*
Franche-Comté (10)	1.23 (1.21-1.25)***	0.97 (0.92-1.03)
Savoie (11)	1.17 (1.13-1.20)***	0.98 (0.87-1.10)

$^{***}P < 0.001$; $^{*}P < 0.05$.

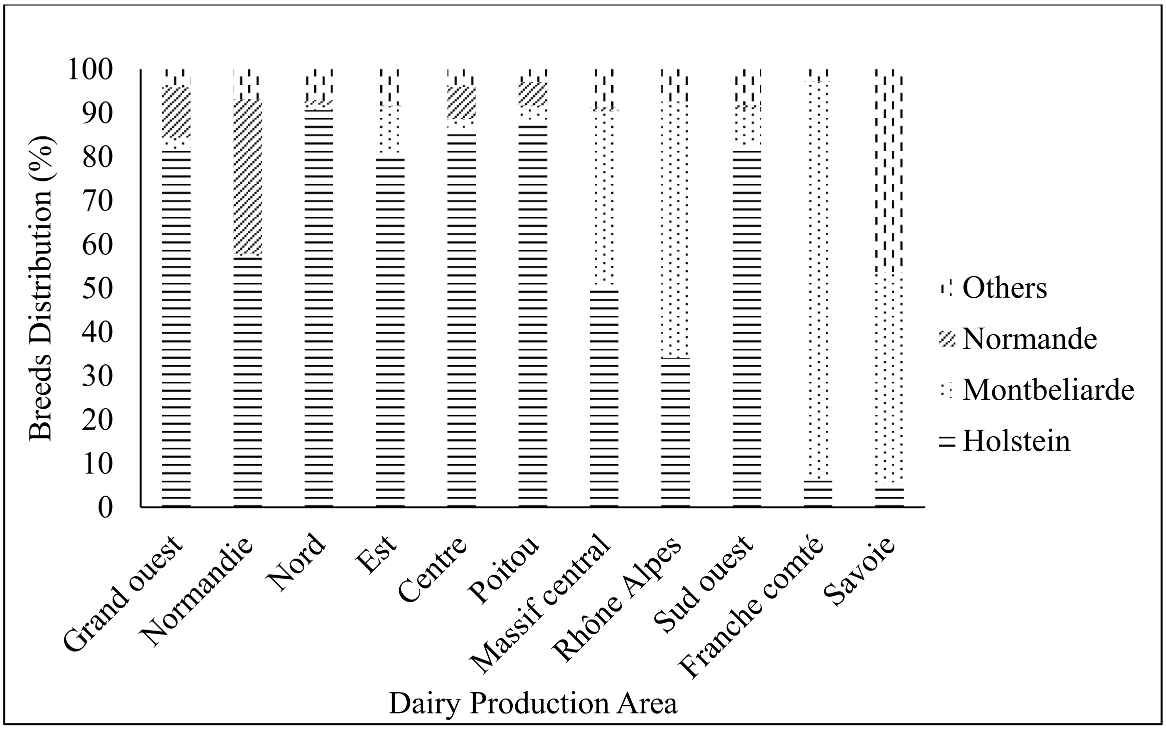

Figure 17. Proportion of each breed of the french dairy cows per dairy production area (DPA)

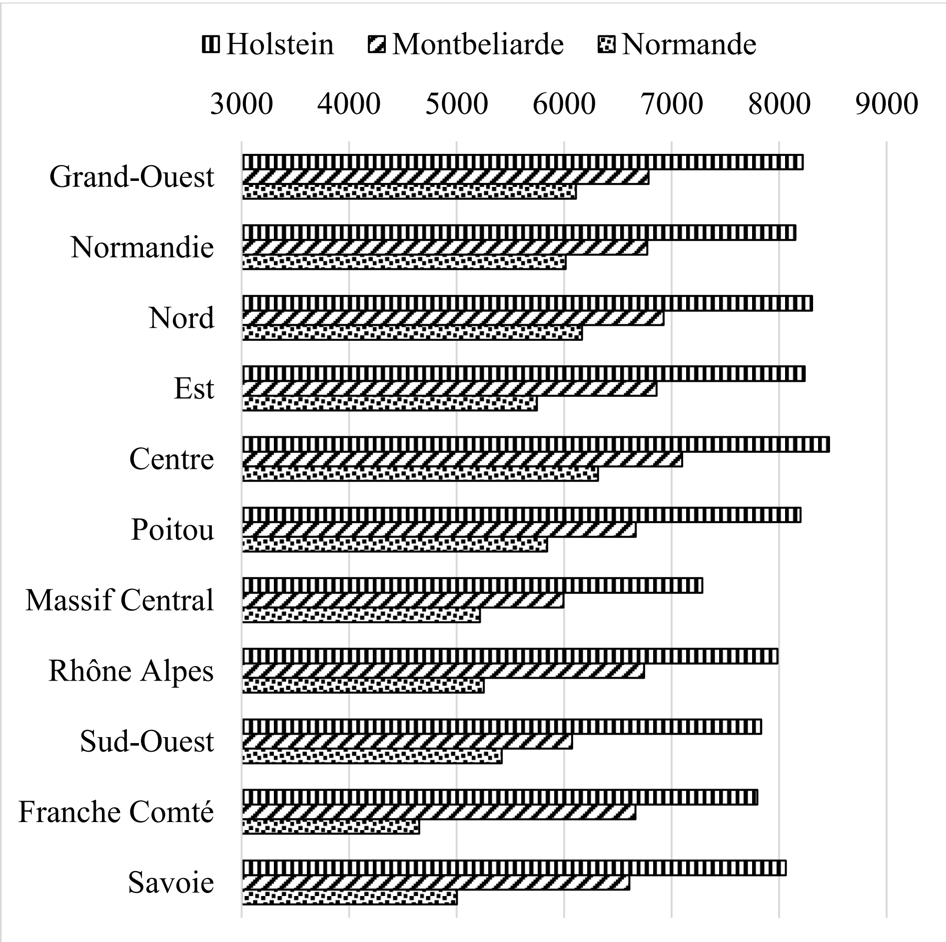

Figure 18. Milk production per breed and per dairy production area (DPA)

2.3.4. Parity and conception

Conception rate linearly decreased when parity increases (Figure 19), whatever all AI or AI1 were considered, and whatever the year of the study (Table 16). Cows in 2nd, 3rd or ≥ 4th parity were less likely to conceive (RR of 0.92, 0.85 and 0.77, respectively) compared to cows in 1st lactation.

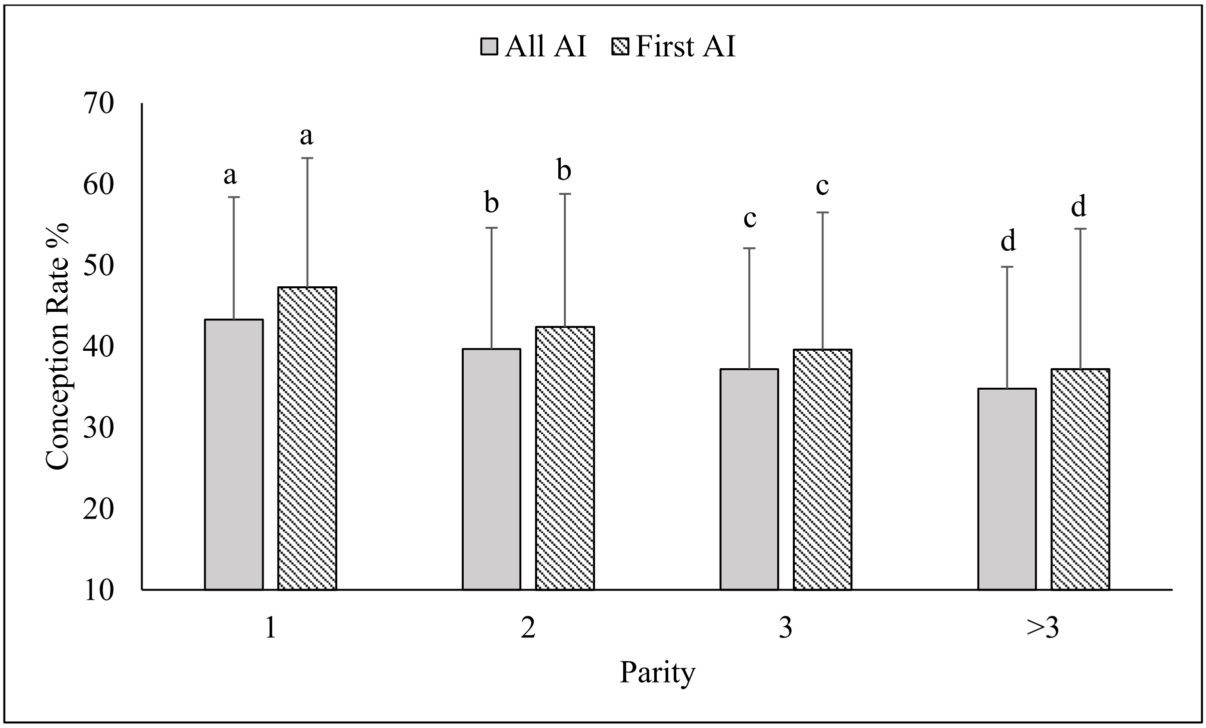

Figure 19. Conception rates (%) at all AI and at AI1 per parity

Table 16. Relative risk (95% CI) of conception at all AI associated with parity (compared to parity 1) within each year of the study

Year	Parity			
	First	Second	Third	Forth and later
2008	Referent	0.91 (0.91-0.92)***	0.85 (0.85-0.86)***	0.78 (0.77-0.79)***
2009	Referent	0.92 (0.92-0.92)***	0.86 (0.85-0.86)***	0.80 (0.79-0.80)***
2010	Referent	0.90 (0.90-0.91)***	0.86 (0.86-0.86)***	0.77 (0.76-0.77)***
2011	Referent	0.91 (0.91-0.91)***	0.85 (0.84-0.85)***	0.78 (0.78-0.79)***
2012	Referent	0.93 (0.92-0.93)***	0.87 (0.87-0.87)***	0.80 (0.79-0.80)***

***$P < 0.001$

2.3.5. Season and conception

Monthly CR at all AI and at AI1 (Figure 20) showed a decrease in the hot season and a peak in Autumn and early Winter. The increase of AI1 in May remain unexplained. Even if trends are similar for each year, the odds of conception may slightly change between years. AI in moderate season (RR of 0.94 to 0.91) and hot season (RR of 0.92 to 0.87) are less likely to succeed compared to Winter. The seasonal pattern of performed AI may be due to calving seasonality (Figure 21), voluntary longer waiting period in summer or less availability of inseminators due to Holidays.

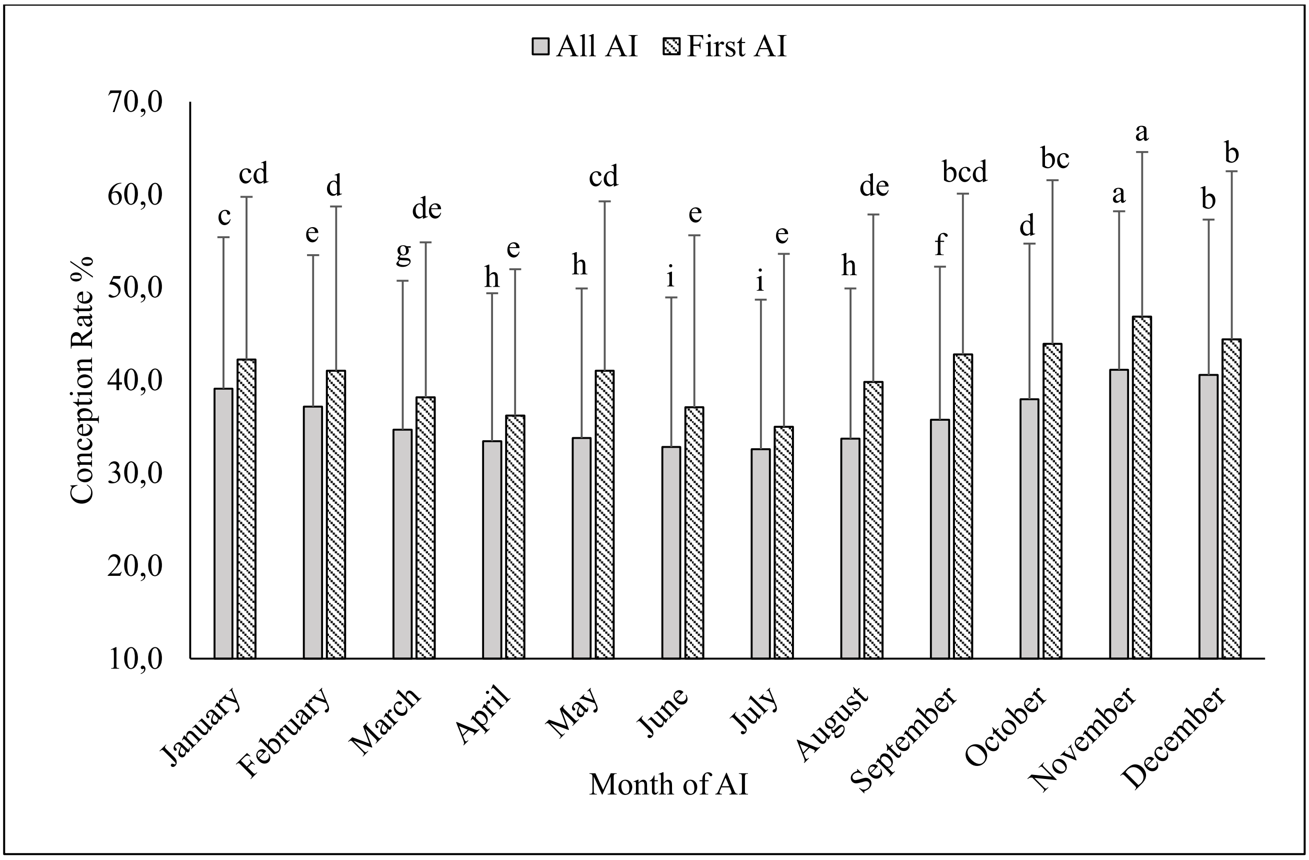

Figure 20. Conception rates (%) at all AI and at AI1 per month

Table 17. Relative risk (95% CI) of conception at all AI and at AI1 associated with moderate and hot seasons (compared to cold season) for cows from all breeds within each year of the study.

Year		Season		
		Cold	Moderate	Hot
2008	All AI	Referent	0.94(0.93-0.94)***	0.88(0.87-0.88)***
	AI1	*Referent*	*0.96(0.95-0.96)****	*0.89(0.89-0.90)****
2009	All AI	Referent	0.94(0.93-0.94)***	0.90(0.90-0.91)***
	AI1	*Referent*	*0.95(0.94-0.95)****	*0.92(0.91-0.92)****
2010	All AI	Referent	0.93(0.92-0.93)***	0.90(0.89-0.90)***
	AI1	*Referent*	*0.94(0.94-0.95)****	*0.91(0.91-0.92)****
2011	All AI	Referent	0.91(0.90-0.91)***	0.90(0.90-0.91)***
	AI1	*Referent*	*0.92(0.92-0.93)****	*0.92(0.92-0.93)****
2012	All AI	Referent	0.92(0.92-0.93)***	0.86(0.85-0.86)***
	AI1	*Referent*	*0.94(0.93-0.94)****	*0.87(0.87-0.88)****

$^{***}P < 0.001$

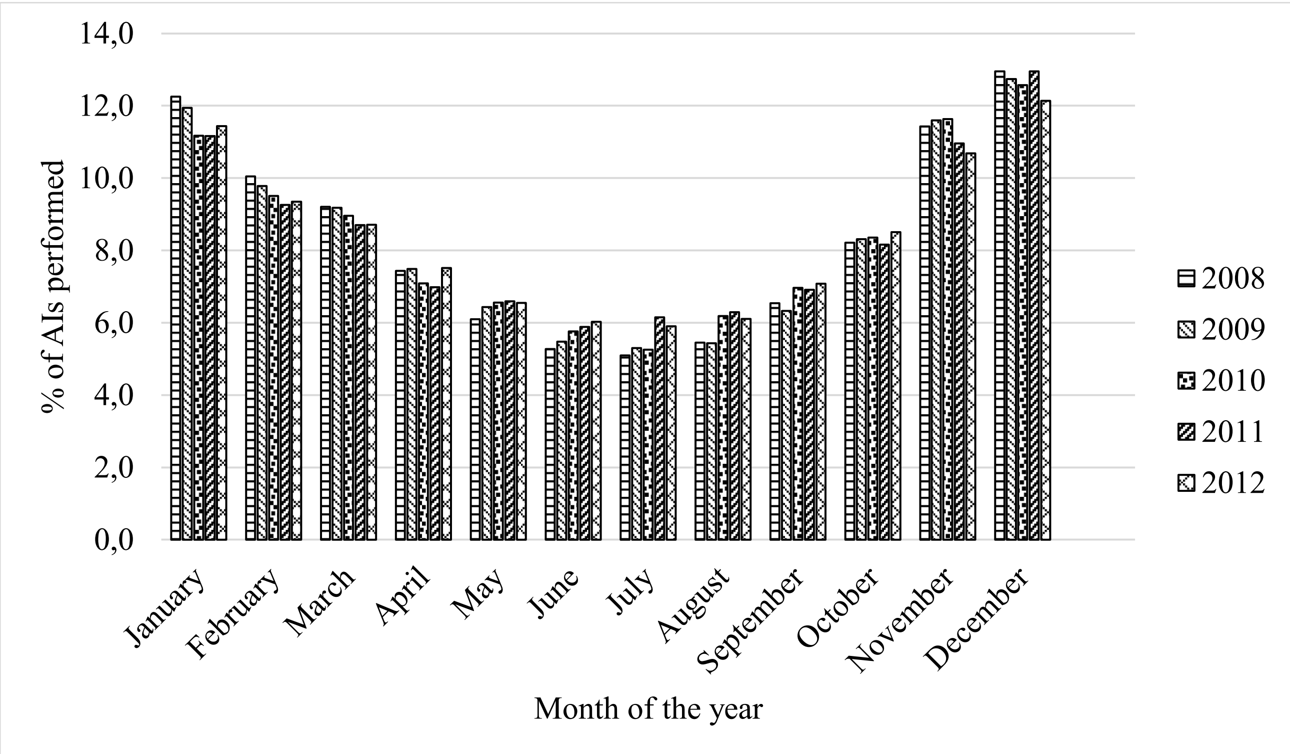

Figure 21. The monthly proportion (%) of performed AI for the 5 years of this study

2.3.6. Days in milk at first service and conception

The mean intervals from calving to AI1 (Table 18) show an earlier AI1 in Montbéliarde and Normande compared to Holstein, but interestingly no association between the success of conception and the date of AI1 was found.

Table 18. Average intervals (days) between calving and AI1 for each year of the study

Breed	Year				
	2008	2009	2010	2011	2012
All breeds	91.5	93.5	94.7	96.0	97.3
Holstein	96.3	98.6	100.0	102.0	103.0
Montbéliarde	80.0	81.2	82.4	82.9	84.2
Normande	82.2	83.9	84.7	86.0	86.7

The Kaplan-Meier survival curves (Figure 22) show that the cumulative probability of conception considerably increased between 50 and 100 DIM, and highlight the high difference in conception success between breeds.

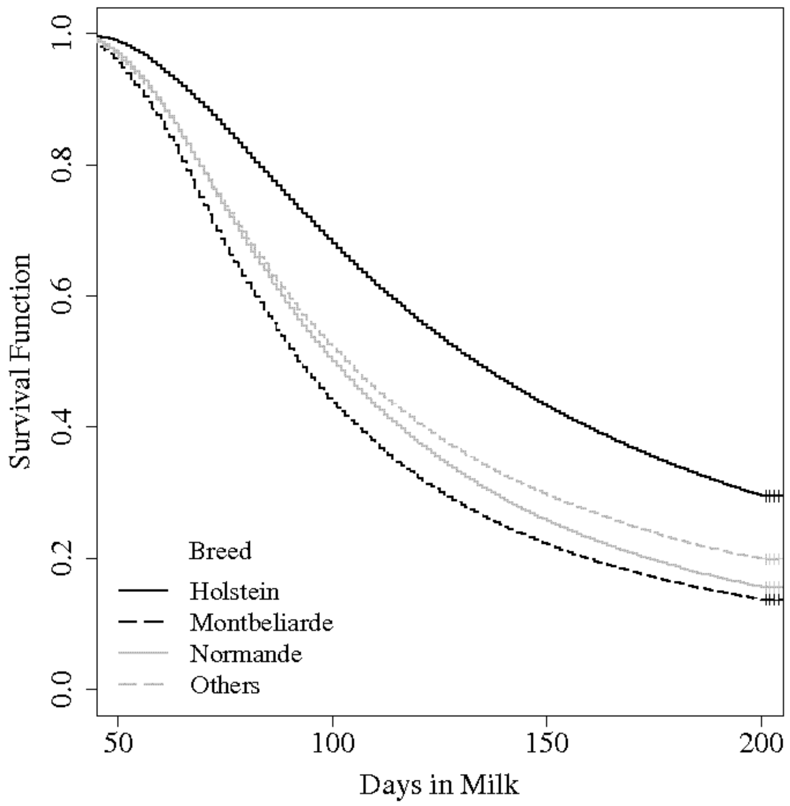

Figure 22. Kaplan-Meier curve of the cumulative probability of AI1 success (survival function) in fonction of days in milk at the time of AI1 and for the three main breeds of french dairy cows

2.3.7. Interval between two consecutive AI and conception

The interval between 2 AI indicates if most cows that didn't conceive are detected already at three weeks or not until 6 or 9 weeks. The average intervals between 2 AI in the present work (Table 19) appear very high for all breeds.

Table 19. Average intervals (days) between 2 consecutive AI for each year of the study.

	Year					
	2007	2008	2009	2010	2011	2012
All breeds						
All cows	56.4	58.7	63.7	63.1	62.6	61.2
Heifers	60.2	62.3	64.1	65.4	67.5	65.7
Lactating cows	56.4	58.7	63.7	63.1	62.6	61.2
Holstein						
All cows	53.2	55.0	59.6	58.4	57.4	55.8
Heifers	53.7	53.8	53.3	51.9	51.6	46.3
Lactating cows	53.1	55.2	60.8	59.7	58.6	57.5
Montbéliarde						
All cows	57.4	61.6	64.7	62.8	60	57.9
Heifers	52.1	53.3	50.6	49.6	50.1	47.4
Lactating cows	58.3	63.7	68.3	66.7	63.2	60.9
Normande						
All cows	51.4	53.9	57.8	56.7	56.8	51.9
Heifers	53.2	52.7	52.0	51.8	51.8	45.4
Lactating cows	51.0	54.2	59.2	58.0	58.1	53.3

The Kaplan-Meier survival curves for intervals between two consecutive AI (Figure 23) showed a high increase in the cumulative probability of conception success when the interval was between 38 and 50 days, and a limited increase in the cumulative probability of conception success when the interval between AI was < 38 days. It suggests that after an unsuccessful AI, a higher probability of conception after 2 estrus cycles compared to one estrus cycle is observed. The cyclicality of the time between 2 AI when looking at the conception remain unexplained (Figure 24).

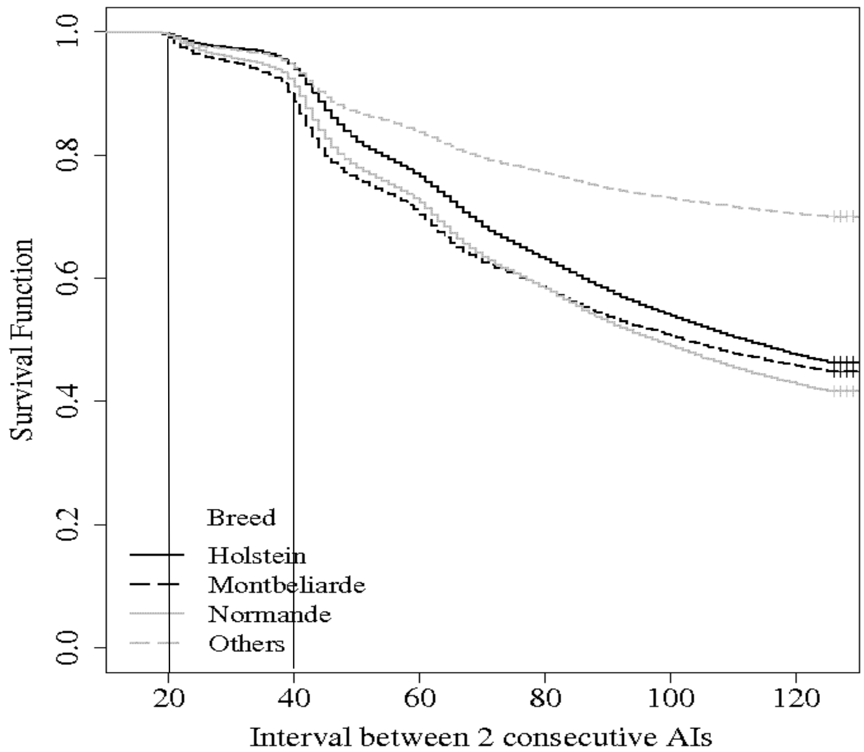

Figure 23. Kaplan-Meier curve of the cumulative probability of AI success (survival function) in fonction of the interval between two consecutive AI and for the three main breeds of French dairy cows.

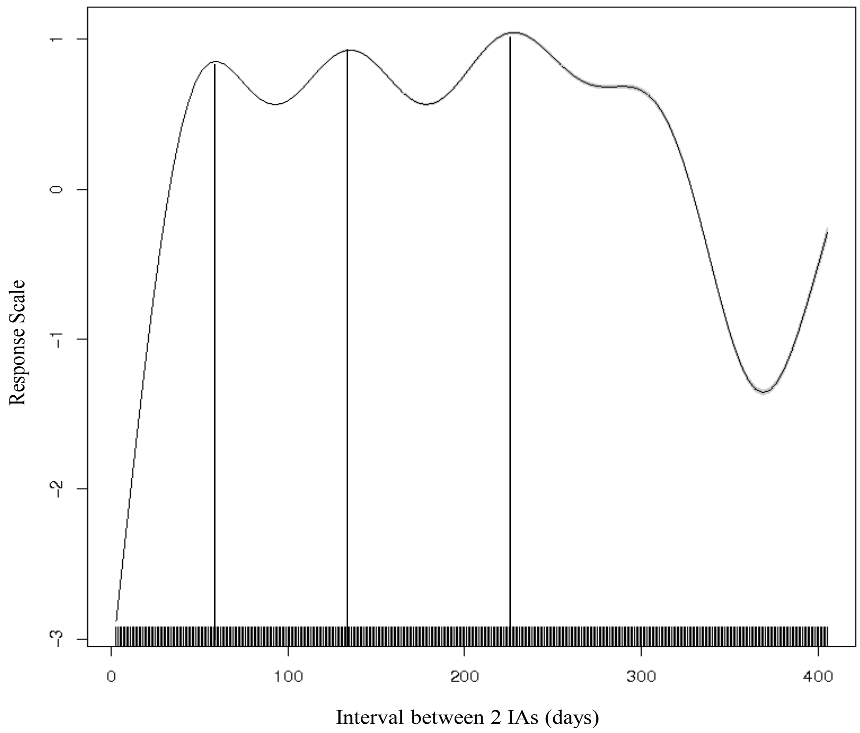

Figure 24. The relationship between the interval (in days) between 2 AI (as smooth functions) and relative values of conception success (response scale).

2.4. Discussion

A decrease in milk, fat and protein yields during the summer months has been shown in the present work, which correspond to the end of the lactation period where cows are being dried off. Several factors could affect the SCC that may exert their influence at the quarter, cow or herd levels, amongst others are infections, parity, stage of lactation, age and season. In the present work, SCC were lowest during the winter and highest during the summer with a peak in July. This in accordance with the general trend already demonstrated (Dohoo and Meek, 1982; Archer et al., 2013), although the mechanism involved is not clear yet. The increase during the summer does not appear to be entirely due to elevated temperatures because attempts to reproduce the effect by putting cows in environmentally controlled chambers and increasing the temperature have not reproduced the same effect (Dohoo and Leslie, 1991). The major factor affecting SCC levels is infection of the mammary gland (Olde Riekerink et al., 2007; Hogan and Smith, 2012). Urea concentrations has been almost stable throughout the year with a slight decrease at the end of the summer (august and September). A similar peak has been reported (Refsdal et al., 1985) and was explained by the presence of grass in animal diets during this season. Urea concentrations have been shown to be higher during the pasture periods because of the high azote concentration in grass.

The stability of CR observed from 2008 to 2012 is in agreement with the slight degradation in the CR (1% per year) observed for the 1999-2004 period in the three principal breeds (Barbat et al., 2010). From 2000 to 2004, the degradation in conception was 2 to 3 points of percentage for Normande and Montbéliarde, and at least 5% for Holstein, but the period 2005 - 2007 displayed a more stable trend, in accordance with the present results. The decrease in fertility of French dairy cows observed in the 2000's was stopped in 2004 (Le Mézec et al., 2010b) where CR stabilized. The interval between calving and the successful AI yet continued to increase. The CR in the present study ranged between 40.6 and 41.7% for cows from all breeds, and this is associated with a relative stability in the yearly 305-days MY within the period 2008-2012.

At a global scale, fertility has been shown decreasing within the 1980's and 1990's for Holstein cattle in Europe and Northern America as reviewed by (Rodriguez-Martinez et al., 2008). However, the same review reported a similar trend to stabilized CR (reported to be around 43%) for Swedish Holstein within 2004 and 2006. In the Netherlands, pregnancy rates decreased by 10.1 point of %, from 55.5% to 45.4% between 1988 and 1998 (Jorritsma and Jorritsma, 2000). While CR to the first service after calving has decreased by 0.4 point of % per year in the 20 years since the mid 70's in the

USA (Beam and Butler, 1999), CR trend to stabilize between 2002 and 2006 at around 30% for Holstein and around 36% for Jersey cows (Norman et al., 2009). In Canada, although a slight decrease in the non-return rate at 56 days from above 69% down to 67% was reported (Bousquet et al., 2004), pregnancy rates have neither increased nor decreased as average production has continued to increase between 1999 and 2011 (LeBlanc, 2013).

Numerous studies noted a tendency toward a decreased reproductive performance within the last 5 decades in most countries (Bousquet et al., 2004; Lucy, 2005) which has been shown to have links with genetic merit for milk production within the same period (LeBlanc, 2010b). However, the inconsistency reported in the present study with the above mentioned general tendency has been reported previously. Despite the continued march of increasing production per cow per year, recent data suggest that the trend in some of the same measures of reproduction has begun to improve in the USA (Norman et al., 2009). It has been assumed that greater milk production is a cause of the apparent decline in reproductive performance over time, because it seems very plausible and both phenomena occurred concurrently (LeBlanc, 2013). Other variables that are obvious candidates to influence the probability and timing of pregnancy such as nutrition, housing, and skilled labor are ignored or acknowledged but not assessed because these data are difficult to obtain. Also, high production may constitute a higher risk in terms of degraded health and fertility only under suboptimal conditions, such as when cows are not fed adequately for high yield (Windig et al., 2005). Among the inconsistency within the relationship between reproduction and milk yield is the fact that high yielding cows may be bred later: in a study, the negative association between conception and MY has been weakened by the introduction of an interaction with DIM (Eicker et al., 1996). Moreover, the genetic correlation between MY and CR in the Normande breed was weak and ranged between -0.07 and -0.10 (Boichard et al., 1999), showing another evidence of inconsistency in the relationship between reproduction and milk yield.

Both the season of calving (Eicker et al., 1996) and the season of AI (Huang et al., 2009) were described as an important factor affecting fertility. Season of calving and of AI are likely to be timely related. The reproductive performance may be affected by different seasonal factors such as feeding (pasture, hay, silage) and climate (temperature, humidity, wind) (Huang et al., 2008). The present results show that conception is more likely to succeed when performed in the cold season of the year. Many studies reported such an association. A large decline in the CR was observed within the months May to June in the USA regardless the milk production level (Huang et al., 2009). The pregnancy rate was 11 percentage units greater in the Winter than during the Summer season (de Vries and Risco, 2005). Heat stress is a consensual risk factor for deterioration of reproduction parameters (Huang et al., 2008). CR was reported to be affected by a high heat load before and after service, in particular if occurring during weeks 3 to 5 before service (Morton et al., 2007). Other management factors such as a switch to more pasture-based systems (Huang et al., 2008) may also be involved in the seasonality of reproductive performance.

Conception rate varied considerably amongst DPA, what is likely to be linked to a combination of several effects. DPA refers to different breeds, milk yields, feeding systems, calving season, … Calvings are distributed all year round in Western France (DPA 1, 2 and 3) and the poorer may be due to a large proportion of AI performed during a less favorable period (Barbat et al., 2010). Even when only Holstein cows were taken into account, DPA 7 and 8 still had higher CR than other regions: lower number of AI in summer compared to other areas, and high differences between feeding systems and milk yields may contribute to this difference. Spatial variations in fertility traits has been extensively described. For instance, in the US, CR was 33% in the Northeast and Southwest, and 26 % in the Southeast (26%) regions (Norman et al., 2009). These differences were reported to be related to differences in the use of synchronization for breeding.

In the present study, parity is shown to be negatively associated with conception. This is in accordance with many studies. In Holstein cows, pregnancy rates at AI1were 42.9, 20.0 and 11.9% for cows in 1st, 2nd and 3rd-4th parities ($P < 0.05$), respectively (Balendran et al., 2008). First parity cows had a significantly greater probability of conception than older cows (Darwash et al., 1997; Friggens and Labouriau, 2010). Cows in third and more lactation had lower CR and longer intervals to first service, but cows in 2nd lactation had reproductive performance equal to those in 1st lactation (Hillers et al., 1984). Increased lactation number was associated with more reproductive disorders and poorer reproductive performance (Coleman et al., 1985). First parity cows had the greatest successful AI1 (43%) compared with other parities (32-39%) ($P < 0.05$) (Inchaisri et al., 2010). Days to last breeding increased by 2 weeks as parity increased in Holstein and calving interval was 12 days shorter for parity 2 than for parities > 5 (Norman et al., 2009). Opposite results were sometimes reported. For instance, no association between age and reproductive performance was found in an old study (Smith and Legates, 1962) and reproductive performance was improved in another paper as lactation number increased: each increase in lactation number was associated with a 4-day decrease in days open (Kinsel and Etherington, 1999).

Inseminating a cow is the first step toward establishing a pregnancy. In herds using AI, the service rate directly reflects estrus detection efficiency because a cow must first be detected in estrus before she can be inseminated. Several studies suggest that subfertility may arise from the inefficiency in estrus detection and thus the failure to perform AI within the desired period (Esslemont and Peeler, 1993). DIM at first service was widely considered to be a major determinant of days open and DIM at first service to be more determined by management practices, specifically estrus detection and voluntary waiting period (VWP), than physiology of the cow (Coleman et al., 1985). This interval may differ between cows according to parity and production levels, but the farmer may alter it based on management decisions (Inchaisri et al., 2011). Our results show that AI has more chance to succeed when performed between 50 and 100 DIM. An interval of 45 to 60 days after calving is often recommended to allow a complete uterine involution and resumption of normal ovarian cyclicality (Inchaisri et al., 2011). Many studies have reported benefits of waiting to inseminate cows until later in lactation, but others stated the contrary. Summarizing seven studies, fertility was 25% for cows inseminated in the first 20 days after calving compared to greater than 60% for cows inseminated after 70 days postpartum (Britt, 1975). Conception was less for cows with less than 50 days to first service (32%) than for cows with over 50 days to first service (49 to 57%) (Hillers et al., 1984). OR for the pregnancy risk were 0.73 and 0.55 ($P < 0.05$) at < 66 days and 66-82 day, respectively, compared to the reference category of 83 to 99 days (Loeffler et al., 1999b).

Many other factors linked to farmer's skill and farm management may influence reproductive performance. Adequate management may improve the health status and increase fertility. For example, following hygienic measures influences considerably the incidence of mastitis which was also associated with factors related to housing, nutrition, and machine milking (Barkema et al., 1999). The feeding system may influence the interval between calving and AI1 (Pryce et al., 1999). Moreover, the analysis of the intervals between two successive AI in the present work shows very long intervals and a high non-return rate. This indicates a significant early embryonic mortality, and / or a deficiency of heat detection.

2.5. Conclusion

The stabilization in CR demonstrated here is in accordance with the tendency reported in France and in some other developed countries within the last 15 years. Although the calculation of CR made in this study is more restrictive than that usually used in the literature, since it doesn't take in account some missed information such as abortion, the reported CR were in agreement with those recently shown for the main three breeds of French dairy cows. The present results are also in agreement with the French and international literature for the seasonal variations of milk components and the remarkable diversity amongst DPA, which needs further work to confirm if this variation could be attributed to breed, breeding system, climate or all of them.

The present descriptive statistics highlight the potentiality and the richness of the database used for the upcoming results. They confirm its usefulness and adequately to evaluate the reproductive status of French dairy cows and to investigate its association with other health disorders defined through the milk components provided by the MCP (SCC, urea, milk and fat contents).

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3. Chapitre 3. Concentrations cellulaires, cétose subclinique et efficacité de la reproduction

Résumé :

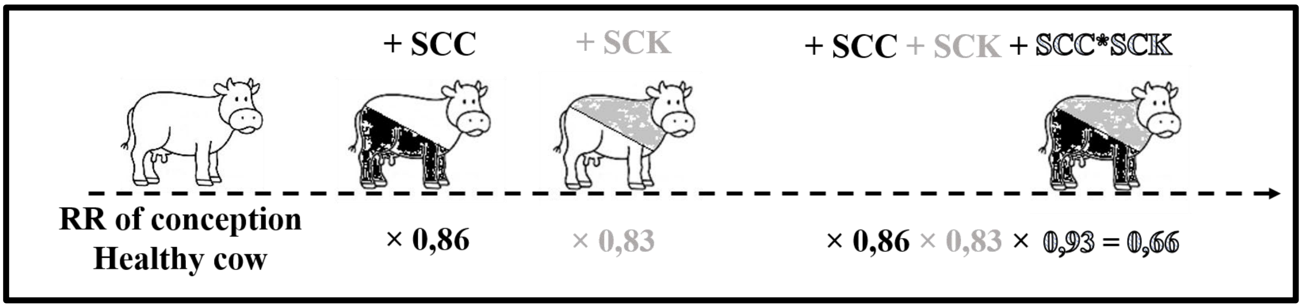
L'objectif de cette partie est de redéfinir les relations entre le statut sanitaire de la mamelle en début de lactation et la fonction de reproduction en présence de la cétose subclinique, à l'échelle de l'animal. Les associations entre l'inflammation mammaire et la réussite de la reproduction sont analysées via la dynamique des CCS autour de l'IA, en intégrant un indicateur individuel de la cétose subclinique.

Les CCS ont été catégorisées comme bas (B) ou haut (H) avant et après chaque IA, et ont été classées en 4 groupes selon la dynamique de ces concentrations autour de l'IA (BB, BH, HB et HH). Le seuil de CCS retenu dans cet article pour distinguer les niveaux bas et haut est de 200 000 cellules/mL. Les indicateurs individuels de cétose subclinique sont construits à partir des variations des taux butyreux (TB) et taux protéique (TP) des contrôles mensuels du contrôle laitier. Un modèle additif général (GAM) univarié expliquant la conception a d'abord été utilisé pour définir les seuils les plus pertinents, sans *a priori*, et un modèle multivarié de régression de Poisson a été ensuite mobilisé, toujours pour expliquer la réussite à l'IA. Une description fine du modèle final est proposée à l'issu de la publication.

Les résultats montrent que la conception d'une vache avec une augmentation de CCS autour de l'IA (CCS < 200 000 cellules/mL avant l'IA et CCS > 200 000 cellules/mL après l'IA) est réduite de 14 % (RR = 0,85-0,87). Ces risques relatifs sont similaires chez les vaches avec un niveau de CCS qui reste élevé autour de l'IA (HH). La réduction de la conception associée à la présence de la cétose subclinique varie entre 3 et 17%, selon la précision de l'indicateur utilisé. Les résultats montrent aussi clairement une interaction entre la cétose subclinique et les mammites dans leur impact sur la conception : la conception est d'autant plus réduite qu'une cétose subclinique est présente chez une vache avec des CCS élevées après l'IA. La baisse de la conception en présence d'une mammite et d'une cétose subclinique par rapport à la situation où il y a seulement une mammite est ainsi 2 fois plus grande. L'effet est d'autant plus grand que la cétose subclinique est présente avant l'IA pendant une période de 40 à 80 jours, montrant que l'effet négatif de la cétose subclinique sur la reproduction est décalé dans le temps.

Une représentation schématique de ces résultats est proposée ci-dessous pour mieux expliquer la force multiplicative de l'interaction : par exemple, les chances de conception chez une vache saine

sont réduites de 14% (RR=0.86) en cas d'inflammation mammaire seule et de 17% (RR=0.83) en cas de cétose subclinique seule. Cependant, la réduction des chances de conception en cas de mammite et de cétose subclinique simultanée est obtenue par la multiplication de ces 2 risques et de celui de l'interaction (ici RR=0.93). Dans cette exemple, les chances de conception en cas de mammite et cétose subclinique simultanée sont réduites de 34% (RR= 0.66) comparé à une vache sans aucune de ces 2 entités.



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High somatic cell counts and changes in milk fat and protein contents around insemination are negatively associated with conception in dairy cows

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The fertility of dairy cows has decreased dramatically worldwide over the last few decades, and several causes of this trend have been reported. Several studies have associated compromised udder health with deteriorating reproduction performance. Subclinical ketosis (SCK) has also been reported to be a risk factor for decreased conception. The objective of the present study was to describe how SCK might interact with the reported association between udder health and conception in dairy cows. Data from the French Milk Control Program and data on 8,549,667 instances of artificial insemination (AI) and their corresponding preceding and subsequent test-days from 5,979,701 Holstein cows were examined over a 5-year period (2008–2012). The effect of udder health was evaluated through a low (L) or high (H) somatic cell count (SCC) before and after AI using a threshold of 200,000 cells/mL, and transformed into four groups (LL, LH, HL, and HH). Three proxies for defining SCK were proposed based on the milk fat and protein content (or their ratio) before AI. Statistical analysis first included a generalized additive model to help define the optimal threshold values. Next, a logistic regression with a Poisson correction was performed. On average, the risk of conception at first AI was reduced by 14% for LH or HH cows (relative risk [and 95% CI] = 0.86 [0.85–0.87]) when the SCC increased or remained high within 40 days before and after AI, relative to LL group. The reduction of conception success associated with SCK (fat and protein contents changes) varied from 3% to 17% depending on the used SCK proxy. Including the interaction term SCC*SCK clearly showed that the association of increased SCC around AI with conception success was modified by the presence of SCK. A cow that already has SCK and experiences an increase in SCC around or after AI exhibits up to 2 times further decrease in conception success compared with a cow with a high SCC and no SCK. In conclusion, this study reinforces the previously described association between intramammary inflammation around or after AI and a decreased rate of conception. These findings highlight how SCK interacts with the above-mentioned relationship by strengthening the negative association between mastitis and conception success. In addition, the present work supports the theory that local inflammation may affect the whole-body response and alter the functions of other organs, such as the reproductive tract.

1. Introduction

Reproductive performance has always been considered a major determinant of dairy herd profitability because of its associations with the amount of milk produced, the

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culling rate, the cost of breeding, and the value of calves. Over the last 15 years, reproductive performance has been declining worldwide [1]. Genetic selection for high milk yield has likely exerted negative effects on reproduction and health, and the demand for high milk production can contribute to a decrease in reproduction ability and reduce the likelihood of pregnancy establishment in high-producing cows [1–3]. Dairy cow reproduction is also highly dependent on peripartum infections [4,5] or nutrition-based disorders [1,6]. Many studies have shown that most of these disorders are risk factors for other disorders. Depending on the studied disorder, the descriptions of epidemiological relationships have varied in comprehensiveness, and the strength of the associations remains imprecise in some cases. It is of great interest to clearly understand the relationships and interactions among different peripartum disorders of dairy cows, particularly for decision-making, prioritization of risk factors in the field, and, more broadly, resource allocation [7]. The indirect positive or negative consequences of these disorders must be accounted for during decision-making.

Recent studies have clearly shown that both the clinical and subclinical mastitis are associated with deteriorated fertility parameters, such as longer intervals from calving to conception, increased services per conception, and increased days open [8–10]. The odds ratio between clinical mastitis or high somatic cell count (SCC) and successful conception has varied from 0.40 to 0.85 [11–14]. The relationship between the severity of mastitis and conception rate appears to have a linear trend. The impact of mastitis on conception appears to be higher for clinical than for subclinical mastitis and for a large SCC increase than for a small increase. The most critical risk period in which mastitis can reduce conception success has been reported to be at the time of [11,13] or within the month after service [8,10].

In addition, most transition dairy cows experience a negative energy balance (NEB) as a consequence of increased energy demands around parturition and decreased dry matter intake shortly before calving. The NEB may be associated with an increased incidence of metabolic disorders, impaired fertility, and other health problems [15]. Both udder health and conception have been shown to deteriorate in cases of subclinical ketosis (SCK), although clear epidemiologic evidence supporting these relationships is scarce [16]. Increased concentrations of both β-hydroxybutyrate (BHBA) and nonesterified fatty acids (NEFA) in blood are often used as markers of SCK, and the most common definition of SCK is a blood BHBA level greater than 1.4 mM during early lactation [17]. Changes in the milk fat and protein contents are additional markers used to identify SCK because lipolysis results in an increased fat content in milk and because a lack of energy in the rumen results in low protein synthesis by ruminal bacteria and a consequent decrease in the protein content [18,19].

The present study aimed to investigate the complex relationship among conception, SCC changes, and metabolic disorders using milk components as indicators of SCK.

2. Materials and methods

2.1. Data and variables

The records from herds in the Milk Control Program (MCP) in France from 2008 to 2012 (inclusive) were provided by France Génétique Elevage (http://www.francegenetique-elevage.fr/). These records included the lactation number, calving date, all test-day milk results, and lactation data (length and production) for all lactations. The French MCP represented 61, 57 and 85% of the herds, cows, and milk produced, respectively. The representation of dairy cows included in the MCP varies among dairy production areas and ranges from 40% to 67%. Measurements of milk urea are optional for farmers registered in the MCP and are conducted on an average of 57.7% of test-days. The records for milk urea during the same time period were provided by the France Conseil Elevage (http://www.france-conseil-elevage.fr/). The French Livestock Institute (http://idele.fr/) provided data on artificial insemination (AI), including the identities of the dams and sires and the dates of all AIs. Data were collected using MySQL software (MySQL, version 5.0, Oracle Corp., Redwood City, CA, USA). Restrictions were implemented so that only Holstein cows and instances of AI prior to 200 days in milk (DIM) were included in the study. The milk yield was adjusted to correspond to a reference lactation period of 305 days (305-day MY). A brief description of the contents of the dataset is shown in Table 1.

For each AI, conception was considered as a binary trait and defined as successful if the instance of AI was followed by a calving 265 to 295 days later. This duration was defined as the average period of gestation for Holstein cows ±15 days, as recommended by the French Livestock Institute. Udder health around AI was investigated through the SCC on the test-day. The SCC has been classified as low (L) or high (H) based on various thresholds but only results for a threshold of 200,000 cells/mL will be detailed in this study. For each AI, cows were categorized into four groups according to the dynamics of the SCC as follows: cows with a low SCC both before and after AI (LL, control group); cows with a low level before and a high level after AI (LH); cows with a high SCC before and a low SCC after AI (HL); and cows with a high SCC both before and after AI (HH). In accordance with the average of 1 test-day per month in France, the test-days within the 40 days before and 40 days after AI were primarily included in the analysis. Test-days from other period, including from 40 to 80 days before and after AI, were also assessed. The SCK status at the time of AI was defined by the milk fat and protein contents of the same test-days in which the SCC was quantified [18,19]. A descriptive schematic of the experimental time periods and test-days included in this study is presented in Figure 1.

2.2. Statistical analysis

Data were analyzed using R (version 2.10.1, 2009–12–14, The R Foundation for Statistical Computing, Vienna, Austria). A two-step statistical approach was used. The optimal thresholds for stratifying the categorical variables included in the final logistic regression (SCC, SCK, DIM, and

Table 1
Data summary for the study population by year.

Item	Year				
	2008	2009	2010	2011	2012
Number of herds	44,124	42,169	39,648	37,905	36,683
Number of cows	1,325,171	1,228,949	1,180,592	1,171,971	1,073,018
% in first lactation	36.4	35.3	36.7	38.1	38.9
% in second lactation	26.5	27.8	26.5	26.7	27.7
% in third lactation	17.8	17.7	18.1	17.1	16.5
% in >third lactation	19.3	19.2	18.7	18.2	16.9
Mean of DIM[a] (days) ± SD	91.0 ± 32.6	92.5 ± 33.1	93.0 ± 33.2	94.4 ± 33.4	95.6 ± 33.7
Mean of 305-day MY[b] (kg)	8187	8187	8383	8608	8476
Number of all AIs[c]	1,909,776	1,761,726	1,686,025	1,674,491	1,517,649
Success rate (%)	36.7	36.5	37.3	36.3	38.1
Number of first AI	1,050,762	983,333	950,582	946,876	867,175
Success rate (%)	39.6	39.6	40.5	39.4	41.5

[a] Days in milk at the time of the first artificial insemination.
[b] Milk yield adjusted to a reference lactation period of 305 days.
[c] Artificial insemination.

305-day MY) were first obtained using a generalized additive model (GAM, the gam package in R). The use of GAMs allowed the nature of the relationship between the response and the set of the explanatory variables to be determined rather than assuming some form of parametric relationship [20]. All continuous explanatory variables were introduced into the model as smooth without any *a priori* assumptions. This approach allowed to determine the most efficient thresholds for stratifying the explanatory variables, such as SCC, DIM, 305-day MY, urea concentration, fat and protein contents, and the fat/protein ratio. Conception was explained with explanatory variables maintained in their continuous distribution. Univariate models were separately generated for all of the above-mentioned explanatory variables, and the multivariate models were constructed by integrating the significant variables ($P < 0.05$) into the same model one by one. No interaction was tested because this was outside the scope of this first step.

The final logistic regression with a Poisson correction was then performed using the nlme package of R. A log link model with a Poisson distribution was proposed as a method to calculate relative risks instead of odd ratios and to simultaneously address the issue of the lack of convergence occasionally observed when performing log binomial regressions [21–23]. The same step-by-step procedure was used to include explanatory variables as that described above for the GAM. This model included the SCC as a categorical variable and SCK as a binary variable, as well as a term representing interaction between these variables. In addition, this model was adjusted for the lactation stage, milk yield, and parity. The urea concentration was not retained because of the consistent lack of statistical significance based on the univariate analysis. This model was applied for the different proxies of SCK created by the GAM. The logistic model, which included herd as a random variable, was applied to the entire dataset from the period 2008 to 2012 or from each year either to the first AI (AI1) following each calving or to all AIs. Although only the results for conception at AI1 are discussed in this article, the results were similar for all AIs.

3. Results

3.1. GAM and definition of thresholds

The thresholds defined by the smoothing function did not differ between the univariate and multivariate models, and only graphs from the univariate model are shown here (Figs. 2 and 3). The results showed high consistency

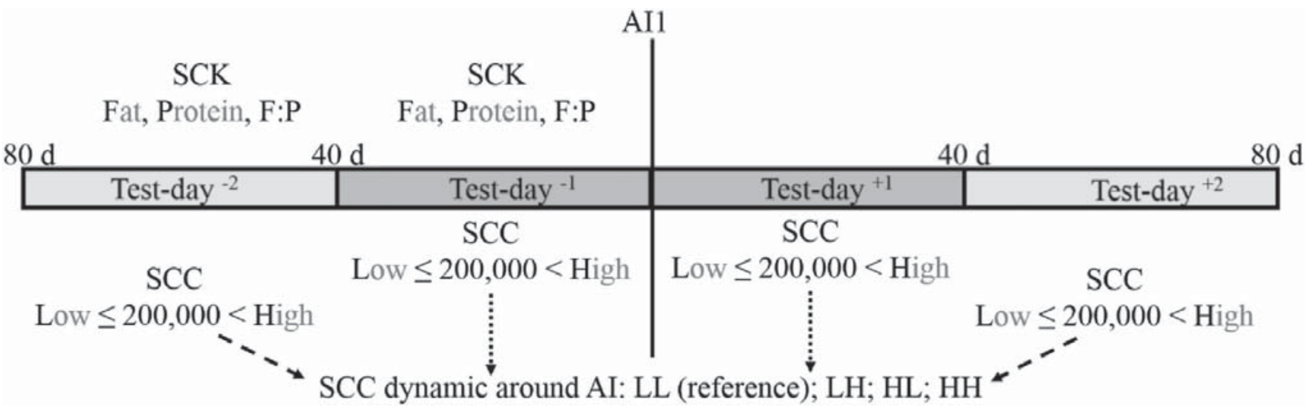

Fig. 1. A schematic representation of the time periods around the first artificial insemination (AI1) and the measurements examined in this study. Several somatic cells counts (SCC) thresholds were tested within 40 days before and after AI1, but only the results for the threshold of 200,000 cells/mL were detailed in this article. A proxy of subclinical ketosis (SCK) was built using the fat and protein contents (mg/kg) or their ratio within 0 to 40 days and within 40 to 80 days before AI1.

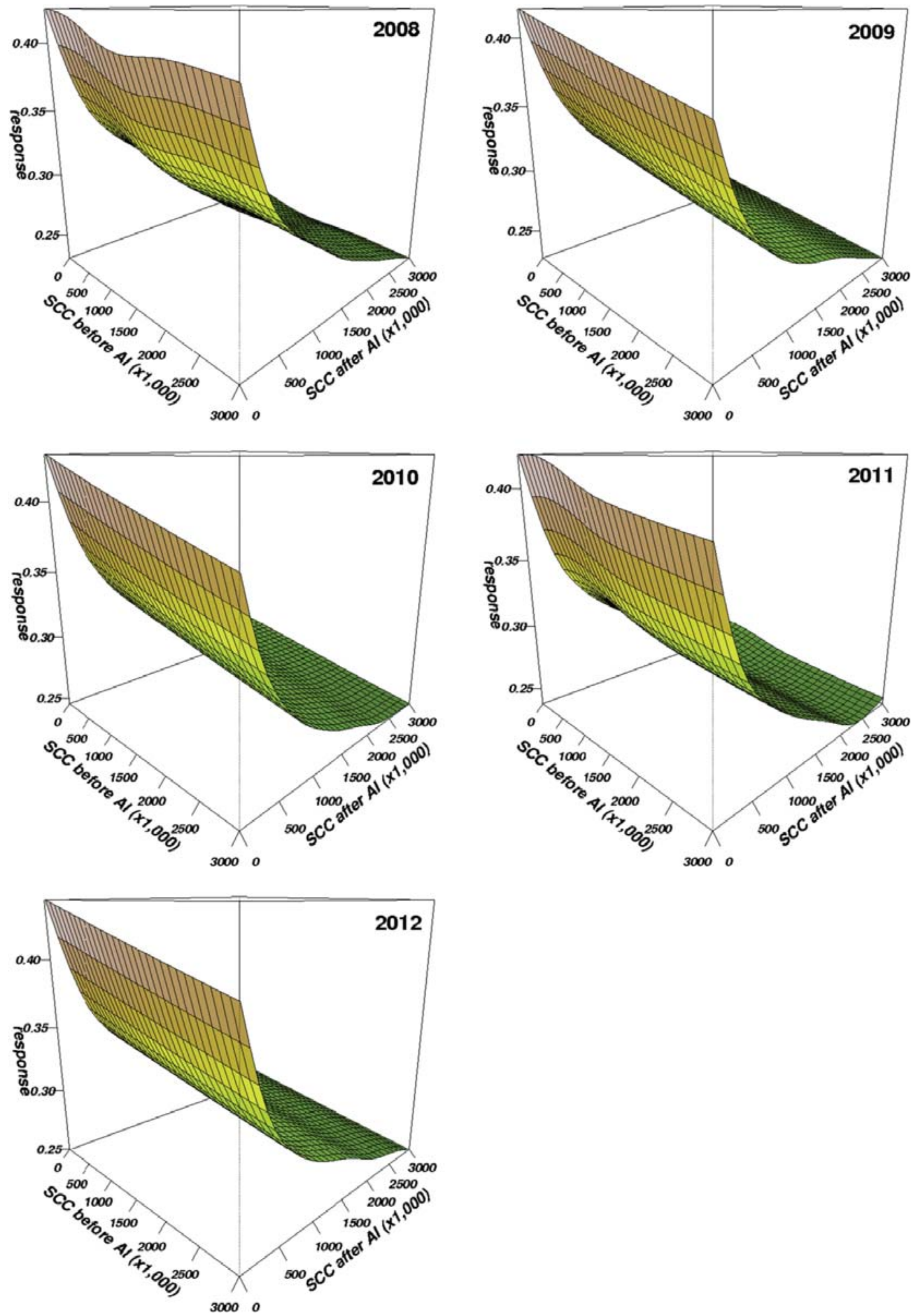

Fig. 2. A three-dimensional representation of the annual relationship between SCC before and after the first AI and conception success (vertical axis). AI, artificial insemination; SCC, somatic cells counts.

between years unrelated to the explanatory variable. A linear but small decrease in conception was associated with an increased SCC before AI (Fig. 2). The conception rate was strongly associated with SCC after AI, with a linear decrease in conception rate up to 400,000 to 500,000 cells/mL followed by an overall very gradual decrease in conception rate as the SCC further increased. Conception success was more closely associated with changes in milk fat content after AI than before AI (Fig. 3). The association between milk protein and conception (Fig. 3) was present both before and after AI, although it was stronger after AI. The results of the GAM led to the retention of

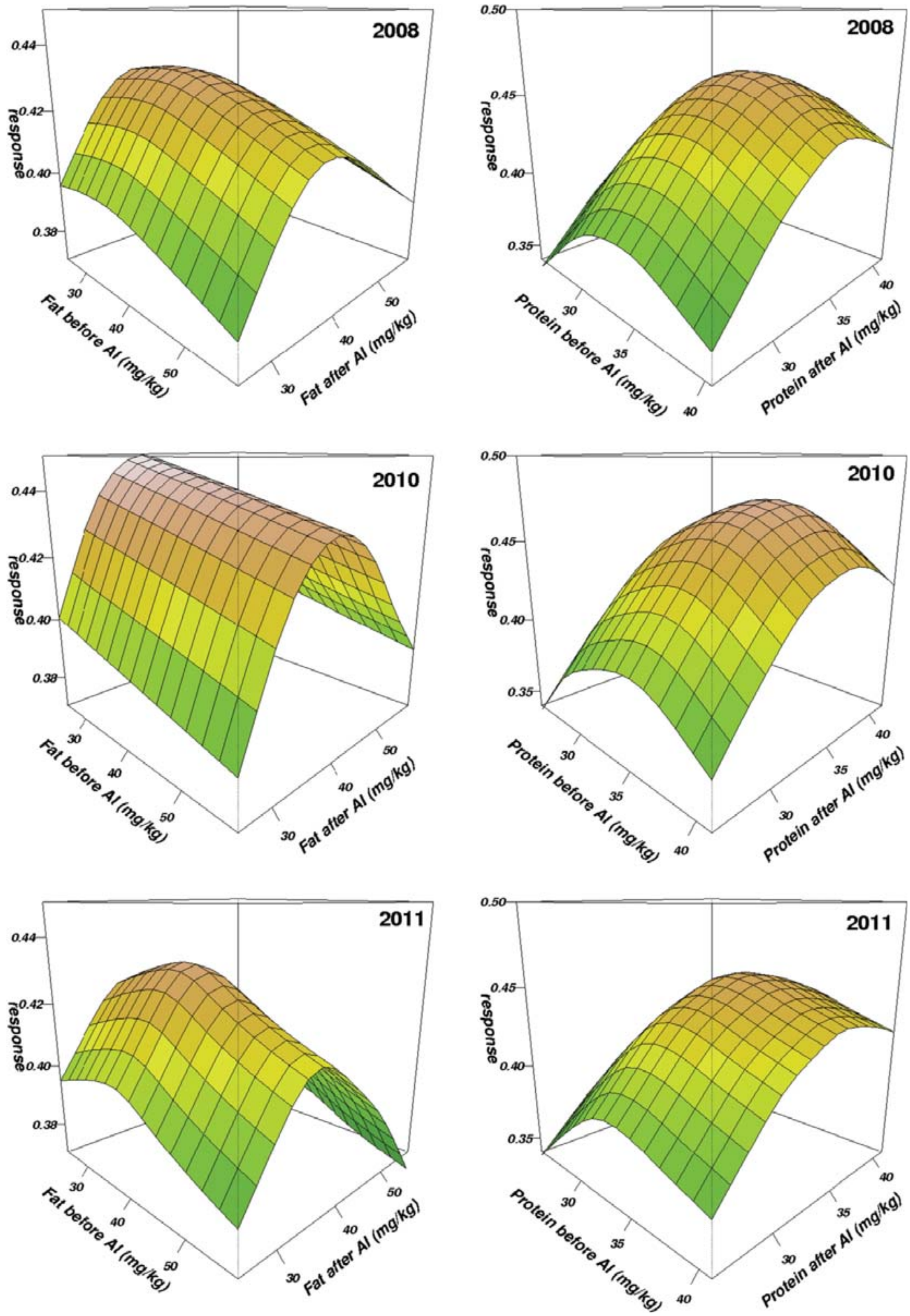

Fig. 3. A three-dimensional representation of the annual relationship between milk fat (left) or milk protein (right) contents before and after the first AI and conception success (vertical axis). Only 3 years of data are reported. AI, artificial insemination.

200,000 cells/mL as the main threshold of the SCC on which to base the associated categorical variable. A threshold of 7000 kg of 305-day MY was used to define highly productive cows. Three proxies of SCK (Table 2) werec implemented according to the associations of fat and protein contents with conception success, as shown in Figure 3.

3.2. The relationship between SCC and conception is strengthened by the presence of SCK

The logistic regression model clearly showed that an increase in the SCC (from low SCC within 40 days before AI1 to a high SCC within 40 days after AI1; LH group) was

Table 2
Definition of subclinical ketosis (SCK) proxies based on milk fat and protein contents (mg/kg) or their ratio and prevalence of SCK according to each corresponding definition.

Proxy code	Definition		Prevalence of SCK
	Presence of SCK	Absence of SCK	
SCK1	Protein before AI[a] <30	Protein before AI ≥30	35%
SCK2	Fat before AI >45 and protein before AI <28	Fat before AI between 35 and 45 and protein before AI ≥32	5%
SCK3	Fat/protein ratio before AI >1.5	Fat/protein ratio before AI between 1.1 and 1.4	10%

[a] Artificial insemination.

associated with a 12 to 17% decrease in conception success at AI1 (Table 3). Similar associations were observed for cows with a high SCC both before and after AI1 (HH group) compared to those with a low SCC before and after AI1 (LL group). Including all AIs in the model, a similar reduction in conception success was observed (results not shown). For alternative thresholds of the SCC, the average decreases in conception success in the LH or HH groups compared to the LL group were 10, 15, 21, and 26% using SCC thresholds of 100,000, 200,000, 400,000, and 800,000 cells/mL, respectively. The conception success at AI1 was significantly higher when performed between 50 and 100 DIM than when performed outside that period, for primiparous cows than for multiparous cows and for low-producing cows compared with high-producing cows (Tables 3–5). No interaction of the above-mentioned variables was significant.

Depending on the SCK proxy, the decrease in the risk of conception at AI1 associated with SCK was between 3 and 17% for AI1 (Table 3). The coefficients of the interactions of SCC with SCK were systematically negative (RR < 1). A cow that already had SCK and experiencing an increase in the SCC around or after AI1 (LH and HH groups; Tables 3–5) exhibited a decrease in the relative risk of conception compared with a cow with high SCC without SCK due to the multiplication of the coefficients of these two events (multiplicative effect), in addition to the inclusion of an interaction term with a coefficient of 0.97 to 0.93. Consequently, the relative risk of conception could be decreased up to 0.66 in cases of SCK concomitant with a high SCC. The effect sizes of the LH*SCK and HH*SCK interactions were greater for certain years, particularly for SCK2, in which the interaction term had a coefficient of 0.90 and even 0.86 (data not shown). Similar results were obtained *via* analysis of all AIs instead of only AI1.

Using the periods of 40 to 80 days before AI1 to define SCK and 0 to 40 days before and after AI to define SCC or 40

Table 3
Relative risk (95% CI) of conception associated with the different explanatory variables for the first artificial insemination (AI1) and for all subclinical ketosis (SCK) proxies considering records from the period of 0 to 40 days around AI1.

Variable	SCK proxy		
	SCK1	SCK2	SCK3
Class of SCC			
LL	Reference	Reference	Reference
LH	0.86 (0.85–0.87)***	0.87 (0.86–0.88)***	0.84 (0.83–0.84)***
HL	0.96 (0.95–0.98)***	0.96 (0.95–0.97)***	0.98 (0.97–0.99)**
HH	0.86 (0.85–0.87)***	0.86 (0.86–0.87)***	0.83 (0.82–0.84)***
SCK			
Without	Reference	Reference	Reference
With	0.93 (0.92–0.93)***	0.83 (0.82–0.85)***	0.97 (0.95–0.99)***
Interaction			
LH* SCK	0.99 (0.97–1.01)	0.95 (0.90–1.00)†	0.99 (0.98–1.01)
HL* SCK	0.99 (0.97–1.01)	1.00 (0.95–1.05)	0.96 (0.94–0.98)***
HH* SCK	0.97 (0.96–0.99)***	0.93 (0.89–0.98)**	0.94 (0.93–0.94)***
DIM[a] (days)			
50–100	Reference	Reference	Reference
<50	0.82 (0.81–0.83)***	0.84 (0.82–0.86)***	0.83 (0.80–0.85)***
100–200	1.00 (0.99–1.00)	0.98 (0.97–0.99)***	1.00 (1.00–1.01)
305-day MY[b] (kg)			
<7000	Reference	Reference	Reference
>7000	0.96 (0.95–0.96)***	0.97 (0.96–0.98)***	0.95 (0.94–0.95)***
Parity			
1st	Reference	Reference	Reference
2nd	0.89 (0.88–0.89)***	0.91 (0.90–0.92)***	0.90 (0.90–0.91)***
3rd	0.83 (0.82–0.83)***	0.85 (0.84–0.86)***	0.84 (0.84–0.85)***
>3rd	0.71 (0.70–0.71)***	0.73 (0.72–0.74)***	0.72 (0.72–0.73)***

***P < 0.001; **P < 0.01; *P < 0.05; and †P < 0.1.
[a] Days in milk at the time of the first artificial insemination.
[b] Milk yield adjusted to a reference lactation period of 305 days.

Table 4
Relative risk (95% CI) of conception associated with the different explanatory variables for the first artificial insemination (AI1) and for all subclinical ketosis (SCK) proxies considering SCC records from the period of 0 to 40 days around AI1 and considering records of SCK from the period of 40 to 80 days before AI1.

Variable	SCK proxy		
	SCK1	SCK2	SCK3
Class of SCC			
LL	Reference	Reference	Reference
LH	0.87 (0.86–0.88)***	0.87 (0.85–0.88)***	0.87 (0.86–0.88)***
HL	0.96 (0.94–0.97)***	0.97 (0.96–0.99)***	0.96 (0.95–0.97)***
HH	0.87 (0.86–0.88)***	0.87 (0.86–0.88)***	0.87 (0.86–0.87)***
SCK			
Without	Reference	Reference	Reference
With	0.95 (0.95–0.96)***	0.87 (0.86–0.88)***	0.93 (0.92–0.94)***
Interaction			
LH* SCK	1.00 (0.98–1.01)	0.97 (0.94–1.01)	1.00 (0.97–1.02)
HL* SCK	1.00 (0.98–1.02)	0.98 (0.93–1.02)	0.99 (0.97–1.02)
HH* SCK	0.98 (0.96–0.99)**	0.96 (0.93–0.99)*	0.96 (0.94–0.98)***
DIM[a] (days)			
50 - 100	Reference	Reference	Reference
<50	0.81 (0.79–0.84)***	0.83 (0.80–0.86)***	0.82 (0.80–0.85)***
100–200	1.00 (1.00–1.01)	1.00 (0.99–1.01)	1.00 (0.99–1.00)
305-day MY[b] (kg)			
<7000	Reference	Reference	Reference
>7000	0.96 (0.95–0.96)***	0.93 (0.93–0.94)***	0.94 (0.94–0.95)***
Parity			
1st	Reference	Reference	Reference
2nd	0.89 (0.89–0.90)***	0.91 (0.90–0.92)***	0.90 (0.89–0.90)***
3rd	0.83 (0.83–0.84)***	0.85 (0.84–0.86)***	0.84 (0.83–0.84)***
>3rd	0.71 (0.70–0.71)***	0.72 (0.71–0.73)***	0.72 (0.71–0.72)***

***P < 0.001; **P < 0.01 and *P < 0.05.
[a] Days in milk at the time of the first artificial insemination.
[b] Milk yield adjusted to a reference lactation period of 305 days.

to 80 days before and after AI1 to define all indicators instead of the period 0 to 40 days led to similar results (Tables 4 and 5).

4. Discussion

The relationship between changes in SCC and conception described in the present study is in accordance with previous studies that reported a negative association between udder inflammation around or after AI and deteriorated reproduction performance [12–14,24]. The comparison of the present results with the literature is limited by the inconsistency of studies on (i) the outcome variables used to qualify reproduction performance; (ii) the definitions of udder health status and SCC variations; (iii) the adjustment of regressions on covariates; (iv) the timing of the events, particularly the period of udder inflammation around AI; and (v) the manner in which the results were reported (odd ratios, relative risks, or mean values among groups). Despite these limitations, previous studies showed a correlation between more severe mastitis (i.e., a large SCC increase compared with a small SCC increase or clinical mastitis compared with only a SCC increase) and a greater decrease in conception rates. Specifically, the percent decreases in conception were approximately 15% to 18% when SCC increased above the threshold of 280,000 cells/mL [12]; 5% to 12% when various SCC thresholds and various periods of increased SCC were considered [13]; and up to 60% when SCC increased above the threshold of 200,000 cells/mL during the conception period [14]. This last result represents an exception among the above-mentioned studies. Previous studies reported that the percent decrease in the conception rate in cases of clinical mastitis was higher, at approximately 13% to 32% depending on the time of the clinical mastitis relative to AI [13] and at 33% to 44% in cases of clinical mastitis occurring after an SCC increase, respectively [24]. Those results are in accordance with the observed decrease in conception success with increased SCC in the present study and with the greater decrease in conception success when the higher thresholds of SCC were tested. Together, these findings reinforce previous but limited studies showing that more severe udder inflammation leads to greater decrease in the conception success [24].

The GAM allows the relationship between two variables to be described without any *a priori* assumptions. The results of the GAM showed a higher impact of SCC change on conception rate after AI than before AI, and this finding is in accordance with the results of logistic regression analysis. These results are also in accordance with the popular finding that conception is more strongly affected by clinical mastitis or increased SCC when these events occur during or only after AI compared to before AI [8,9,11,13,25]. One exceptional result was obtained in a study focusing on SCC only before AI [26], and another exceptional result was produced in the context of a definition of mastitis based on culture findings, among other factors [11]. The similar relative risks of an increased SCC at the time of AI (LH group) and a high SCC both before and after AI (HH group) observed in the present study confirm that conception is dramatically impacted by udder health status following AI.

Subclinical ketosis is a risk factor for deteriorated reproductive performance, including a reduced first service

Table 5
Relative risk (95% CI) of conception associated with the different explanatory variables for the first artificial insemination (AI1) and for all subclinical ketosis (SCK) proxies considering records from the period of 40 to 80 days around AI1.

Variable	SCK proxy		
	SCK1	SCK2	SCK3
Class of SCC			
LL	Reference	Reference	Reference
LH	0.84 (0.83–0.85)***	0.84 (0.83–0.85)***	0.84 (0.83–0.84)***
HL	0.98 (0.97–0.99)***	0.98 (0.97–0.99)***	0.98 (0.97–0.99)**
HH	0.83 (0.82–0.84)***	0.84 (0.83–0.85)***	0.83 (0.82–0.84)***
SCK			
Without	Reference	Reference	Reference
With	0.96 (0.95–0.96)***	0.87 (0.86–0.89)***	0.94 (0.93–0.94)***
Interaction			
LH* SCK	0.99 (0.97–1.00)†	0.94 (0.91–0.97)***	0.97 (0.95–0.99)***
HL* SCK	0.98 (0.96–1.00)*	1.00 (0.97–1.03)	0.99 (0.98–1.01)
HH* SCK	0.97 (0.96–0.99)***	0.93 (0.90–0.96)***	0.96 (0.94–0.98)***
DIM[a] (days)			
50 - 100	Reference	Reference	Reference
<50	0.82 (0.79–0.84)***	0.83 (0.80–0.85)***	0.83 (0.80–0.85)***
100–200	1.01 (1.00–1.01)***	1.00 (1.00–1.01)	1.00 (1.00–1.01)
305-day MY[b] (kg)			
<7000	Reference	Reference	Reference
>7000	0.95 (0.95–0.96)***	0.93 (0.93–0.94)***	0.95 (0.94–0.95)***
Parity			
1st	Reference	Reference	Reference
2nd	0.90 (0.89–0.90)***	0.92 (0.91–0.92)***	0.90 (0.90–0.91)***
3rd	0.84 (0.83–0.84)***	0.86 (0.85–0.87)***	0.84 (0.84–0.85)***
>3rd	0.72 (0.72–0.73)***	0.73 (0.72–0.74)***	0.72 (0.72–0.73)***

***P < 0.001; **P < 0.01; *P < 0.05; and †P < 0.1.
[a] Days in milk at the time of the first artificial insemination.
[b] Milk yield adjusted to a reference lactation period of 305 days.

conception rate [16], as well as for mastitis [27,28]. To the authors' knowledge, no peer-reviewed quantification of the link between SCK and increased SCC or clinical mastitis is available [16]. Despite the proxy used in place of a consensus definition of SCK (based on BHBA or NEFA), the present study reported a decrease in conception success ranging between 3% and 17% in cows exhibiting SCK. The proxies of SCK used in this study were defined by the GAM and are in accordance with the criteria generally used in the field and reported in the literature [18,19]. The sensitivity and specificity of these indicators remain moderate [18]. The fact that the effect of SCK remained similar between the period of 0 to 40 days before AI and the period of 40 to 80 days before AI is in accordance with previous results showing a significant decrease in conception associated with the NEB that occurs 80 to 60 days before AI [29].

No previous study has reported an interaction between SCK and SCC that affects conception. This is the first report demonstrating that SCK may interact with the relationship between SCC and conception. For all the definitions of SCK proxies used here, the presence of SCK magnified the negative association between high or increased SCC and conception success. Further work is needed using a definition of SCK that better aligns with the consensus definition.

The variability in the coefficients of the interaction term among the different years (in particular for SCK2) observed in the present study may originate from the changes in the prevalence of SCK between the years of the study. The validity of an interaction between the SCC and SCK is strengthened by the difference in the coefficient for the SCC variable when the model without interaction was alternatively used to analyze the populations with and without SCK (results not shown).

The mechanism underlying the relationship described in the present work remains unclear. However, a positive genetic correlation between udder health and conception has been reported in several studies [30,31]. The mechanisms by which clinical or subclinical mastitis potentially interacts with conception may include alterations in endocrine profiles and follicular development, inhibition of LH and FSH *via* cytokine release, and intrauterine embryonic survival [32]. Whether the link between mammary inflammation and reproduction failure may involve changes in the cytokine network is also uncertain. The well-described changes in immune function during SCK [33,34] are also in accordance with the SCC*SCK interactions that explain conception success in the present work. This is also in accordance with a recent study showing that ewes from high-SCC genetic lines have increased NEFA and BHBA concentrations after energy restriction, which reinforces the hypothesis of a genetic association between mastitis susceptibility and energy metabolism [35].

From a practical perspective, the present results clearly support the concept of loser cows [36,37]. As a result of the strong associations among disorders, an ill animal is more likely to be affected by other disorders, thereby creating a vicious cycle. This characteristic is of importance in allocating resources and calculating the indirect costs of disorders [7]. However, the present results should not be used as a criterion to select cows to be served at this point.

First, cows with mastitis before AI and no mastitis within one or 2 months after AI were not highly affected (HL cows), and there is no justification for excluding cows with mastitis before estrous from being served unless the mastitis is chronic. More broadly, excluding cows from being served because they were predicted to have a low rate of conception success (e.g., with SCK) improves the indicator "number of AIs per conception," but may simultaneously reduce the calving interval or increase culling and turnover. The cost of AI (induced labor) remains the key parameter for deciding whether to serve a given cow in this situation.

4.1. Conclusion

The present work is in accordance with previous studies that demonstrated the negative impact of mastitis at the time of AI on conception success. These results also reinforce previous but limited studies showing that the more severe udder health infection or inflammation exacerbates the decrease in conception success. A high SCC after AI has a much greater negative association with decreased conception success than a high SCC before AI. An increase in the SCC was clearly associated with a 12% to 17% decrease in the conception success. A cow that already had SCK and experiences an increase in SCC at the time of or after AI exhibits a multiplicative decrease in the conception success compared with a cow with a high SCC without SCK. However, further research is needed to improve the quantification of these interactions using more accurate definitions of SCK.

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Supplemental data:

Statistical model design:

First, the relationship between the conception success was explained by each variable of interest (SCC, urea, fat and protein contents) kept in its continuous distribution through a univariate regression model. These models were tested for values of the indicated variables before and after AI. Also, univariate models were used to investigate the relationship between conception success and the co-variables commonly used in the field such as 305-d MY, DIM, parity.

Second, these variables were transformed into categorical (when necessary) using the thresholds defined by the GAM results as explained in the article. Only variables that significantly affected the response variable were kept in the final model. To confirm the viability of the constructed model, the fitting of the regression model was made through a stepwise regression with a combination of the forward and backward selection techniques. In this step, all candidate variables in the model are checked to see if their significance has been reduced below the specified tolerance level. If a non-significant variable is found, it is removed from the model. The variables retained in the proposed model were in accordance with the adjustment of co-variables usually used in this kind of studies.

An example of the final logistic regression model is explained below:

$Y_{hjkl} = \beta0 + \beta1 \times SCC_{cat} + \beta2 \times SCK_{p} + \beta3 \times DIM_{h} + \beta4 \times MY_{j} + \beta5 \times Parity_{k} + \beta6 \times SCC_{cat}{:}SCK_{p} + Herd_{l} + e_{hjkl}$,

where

Y_{hjkl} is the success of AI_i at the SCC category and the presence of subclinical ketosis for each cow in herd $_l$ in parity $_k$ with a milk yield class $_j$ and in DIM class $_h$;

β0 is the overall mean success probability of AI_i;

β1 to β6 are the regression coefficients of each component of the regression model;

SCC_{cat} is the fixed effect of the category $_{cat}$ of SCC (cell/mL) on the success of AI_i ($_{cat}$ = as defined in figure 1 of the article);

SCK_p is the fixed effect of the proxy $_p$ of subclinical ketosis SCK on the success of AI_i ($_p$ = as defined in table 2 of the article);

DIM_h is the fixed effect of class $_h$ of days in milk at AI_i the success of AI_i ($_h$ = Class 1 (ref): 50-100 DIM; Class 2: <50 DIM; and Class 3: 100-200 DIM);

MY_j is the fixed effect of class $_j$ of 305-days milk yield MY on the success of AI_i ($_j$ = Class 1 (ref): < 7000 kg; Class 2: > 7000 kg);

$Parity_k$ is the fixed effect of class $_k$ of parity the success of AI_i ($_k$ = Class 1 (ref): parity 1; Class 2: parity 2; Class 3: parity 3; and Class 4: parity ≥4);

$SCC_{cat}:SCK_p$ is the fixed effect of the interaction between the category cat of SCC and the proxy $_p$ of subclinical ketosis SCK on the success of AI_i;

$Herd_l$ is the random effect of herd $_l$;

e_{hjkl} is the residual error.

4. Chapitre 4. Déséquilibres azotés, cétose subclinique et efficacité de la reproduction

Résumé :

L'objectif poursuivi ici est d'analyser l'association dynamique entre une urée du lait faible par rapport au moment de l'IA et la fertilité, ainsi que l'effet d'une variation de la concentration de l'urée autour de l'IA.

La concentration en urée a été catégorisée comme intermédiaire (I ; 250 et 450 mg/kg) ou basse (B ; < 150 mg/kg) avant et après l'IA. L'indicateur individuel de cétose subclinique (TB > 45 mg/kg et un TP < 28 mg/kg) a été défini avec les taux avant l'IA.

Les risques relatifs associés aux valeurs basses de l'urée du lait ont d'abord été calculés pour des valeurs d'urée bas soit avant, soit après l'IA. Un niveau d'urée bas avant l'IA n'était pas associé à une différence de conception. Une association significative mais de faible portée biologique a été observée entre l'urée après IA et la conception : le risque relatif [IC95%] de la conception lors de valeurs de l'urée basses après IA est de 0,96 [0,94‑0,99]. La réduction de la conception associée à la présence d'une cétose subclinique a été similaire à celle observée dans la section précédente. L'interaction entre l'urée et la cétose subclinique n'est pas significative.

La combinaison des niveaux d'urée avant et après IA a permis d'obtenir 4 groupes (II, IB, BB, BI). Les chances de conception sont de 5 à 9% plus faibles (Risque relatif [IC95%] = 0.91 [0.87‑0.96]) pour une diminution de l'urée autour de l'IA (IB) comparé à une urée intermédiaire et stable autour de l'IA (II). Ceci montre l'intérêt de la stabilité de la concentration de l'urée dans le lait chez la vache laitière, préconisant à la fois l'absence d'augmentation ou de baisse forte de sa concentration autour de l'IA.

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Changes in milk urea around insemination are negatively associated with conception success in dairy cows

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ABSTRACT

Dietary protein levels are a risk factor for poor reproductive performance. Conception is particularly impaired in cases of high blood or milk urea. The objective of this study was to investigate the association between conception and low milk urea or changes in milk urea around artificial insemination (AI). Data were obtained from the French Milk Control Program for a 4-yr period (2009–2012). Milk urea values between 250 and 450 mg/kg (4.3 and 7.7 m*M*) were considered intermediate (I), and values ≤150 mg/kg (2.6 m*M*) were considered low (L). Milk urea values before and after each AI were allocated into 4 classes representing the dynamics of milk urea (before-after; I-I, I-L, L-I, and L-L). Subclinical ketosis was defined using milk fat and protein contents before AI as proxies. A logistic regression with a Poisson correction and herd as a random variable was then performed on data from Holstein or all breeds of cows. The success of conception was decreased [relative risk (95% confidence interval) = 0.96 (0.94–0.99)] in low-urea cows compared with intermediate-urea cows after AI; no significant association was found for urea levels before AI. When combining data on urea before and after AI, I-L urea cows exhibited a 5 to 9% decrease in conception compared with I-I urea cows, and L-I urea cows showed no difference in conception success compared with I-I urea cows. A decreased conception success for L-L urea cows compared with I-I urea cows was observed for the analysis with cows of all breeds. This work revealed that a decrease in urea from intermediate (before AI) to low (after AI) is a risk factor for conception failure. Surveys of variation in milk urea in dairy cows close to breeding are highly recommended.
Key words: dairy cow, milk urea, conception

INTRODUCTION

Efficient nutritional management of dairy cattle at calving is one of the most important factors in sustainable dairy farming. It has important effects on the health and reproductive performance of dairy cows. Farmers adopt various nutritional strategies to meet high production requirements while minimizing feed costs and maximizing economic returns. Cows in the early postpartum period experience negative energy balance when their energy requirements exceed their dietary intake (Bauman and Currie, 1980; Bell, 1995). Negative energy balance remains a major challenge for the dairy industry, and extensive literature is available on its physiology, strategic management, and effects on health and performance (Beam and Butler, 1999; Wathes et al., 2003; Raboisson et al., 2014). Protein supply presents a major dietary challenge for postpartum and lactating cows. High-protein diets are widely used during early lactation to stimulate high milk production. However, such diets may be deleterious for reproductive performance, especially when rumen-degradable or rumen-undegradable protein exceeds the cow's requirements (Butler, 1998). In contrast, diets with low levels of proteins are often provided to beef cows, which may be fed only hay or grazed on old-stage pastures. Low-protein diets are occasionally used in dairy herds, and the number of herds with continuous low bulk milk urea or high variations in milk urea is increasing (authors' observations).

Urea is the metabolic end product of protein catabolism in the body (Butler, 1998). Blood urea concentration is a sensitive indicator of protein metabolic efficiency (Kenny et al., 2002). As it circulates through the blood, concentrations of urea equilibrate into all body fluids because of its small molecular size and neutral charge. The level of blood urea is highly correlated with that of milk urea, and both can be used to evaluate the nitrogen nutritional status of the animals (Oltner and Wiktorsson, 1983; Baker et al., 1995).

The link between excess dietary protein, particularly RDP, and the fertility of dairy cows has been widely

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studied. Many studies have reported a negative association between high dietary protein levels and reproductive performance (Butler et al., 1996; Chaveiro et al., 2011). Other studies have found that dietary protein level has no effect on fertility or conception, although it is highly correlated with milk and blood urea (Barton et al., 1996; Dawuda et al., 2002; Laven et al., 2004). Cows can adapt to continuous high dietary protein without any decrease in reproductive performance, but an increase in dietary protein is a known risk factor for infertility (Westwood et al., 1998).

Few studies have investigated the association between low dietary protein and reproductive performance. The interval between calving and the first service was found to be longer in herds with urea <4.5 m*M* compared with herds with urea between 4.5 and 5 m*M* in one study (Gustafsson and Carlsson, 1993) and in herds with low urea concentration in another study (Carlsson and Pehrson, 1993). Cows with low blood urea (<2.5 m*M*) and glucose also had poor fertility (Miettinen, 1991). These results have limited value because these analyses (1) were performed using the mean herd values (Gustafsson and Carlsson, 1993); (2) included low numbers of cows (Miettinen, 1991); and (3) measured urea only once at a fixed date without distinguishing before and after insemination. The only work that has analyzed the effect of changes with respect to the date of AI reported the opposite results, with increased conception success with low (<4.5 m*M*) and high (>6.5 m*M*) urea levels before AI compared with moderate urea values (Godden et al., 2001).

The objective of this study was to investigate the association between low milk urea concentrations and conception in dairy cows, with a special focus on the dynamic of urea levels around AI.

MATERIALS AND METHODS

Data and Variables

The records from herds enrolled in the Milk Control Program (**MCP**) in France from 2009 to 2012 (inclusive) were provided by France Livestock Genetics (http://www.francegenetique-elevage.fr/). Records included lactation number, date of calving, all test-day milk results, and lactation data (length and production) for all lactations. The MCP included 61, 57, and 85% of the herds, cows, and milk produced in France, respectively. The coverage rate of dairy cows included in the MCP varied across dairy production areas (from 40 to 67%). Measuring milk urea is optional for farmers enrolled in the MCP and is done on an average of 58% of test-days. The records for milk urea during the same period were provided by France Conseil Elevage (http://www.france-conseil-elevage.fr/). The French Livestock Institute (http://www.idele.fr/) provided data on AI. These data included the identity of the dams, dates of all AI, and the identity of the sires. Data were collected using MySQL software (version 5.0, Oracle Corp., Redwood City, CA). Milk urea data were gathered from the test-day database. Only AI at DIM <200 were included, and milk yield was adjusted to correspond to a reference lactation period of 305 d (**305-d MY**).

The final data set contained milk urea and production data from the closest test-days before and after each AI. The periods between the previous test-day and the AI and between the AI and the following test-days were restricted to <40 d (Figure 1). This limited the exclusion of AI for early and late test-days, in accordance with an average of one test-day per month in France. The average interval between AI and both precedent and subsequent test-day was 17 d. A brief description of the final data set for each year of the study is reported in Table 1.

A proxy of subclinical ketosis (**SCK**) was constructed using fat and protein contents from the closest test-day before AI. This proxy was defined as fat content >45 mg/kg and protein content <28 mg/kg, as previously described (Albaaj et al., 2016). This definition also accounts for the criteria that are generally used in the field and reported in the literature (Duffield et al., 1997; Heuer et al., 2001).

For each AI, conception was considered a binary trait and defined as successful if it was followed by a calving after 265 to 295 d. This duration was defined as the average period of gestation ± 15 d, as recommended by the French Livestock Institute (www.idele.fr). Milk urea was first examined as a continuous variable to investigate the shape of its association with conception success. It was then classified as intermediate (**I**) for values between 250 and 450 mg/kg (4.3 and 7.7 m*M*) or low (**L**) for values ≤150 mg/kg (2.6 m*M*). These thresholds were defined using a nonparametric model (see Statistical Analysis). They are also in accordance with previous results in this field (Refsdal et al., 1985; Pehrson et al., 1992; Carlsson and Pehrson, 1994). The lower threshold of optimal milk urea concentrations reported in these studies was 4 m*M* (233 mg/kg). Another study reported this threshold to be between 175 and 220 mg/kg (Kirchgessner et al., 1988).

Then, for each AI, cows were categorized according to the dynamic of milk urea levels into 4 classes: cows with intermediate levels before and after AI (**I-I**; control group), cows with intermediate levels before and low levels after AI (**I-L**), cows with low levels before and intermediate levels after AI (**L-I**), and cows with low levels before and low levels after AI (**L-L**).

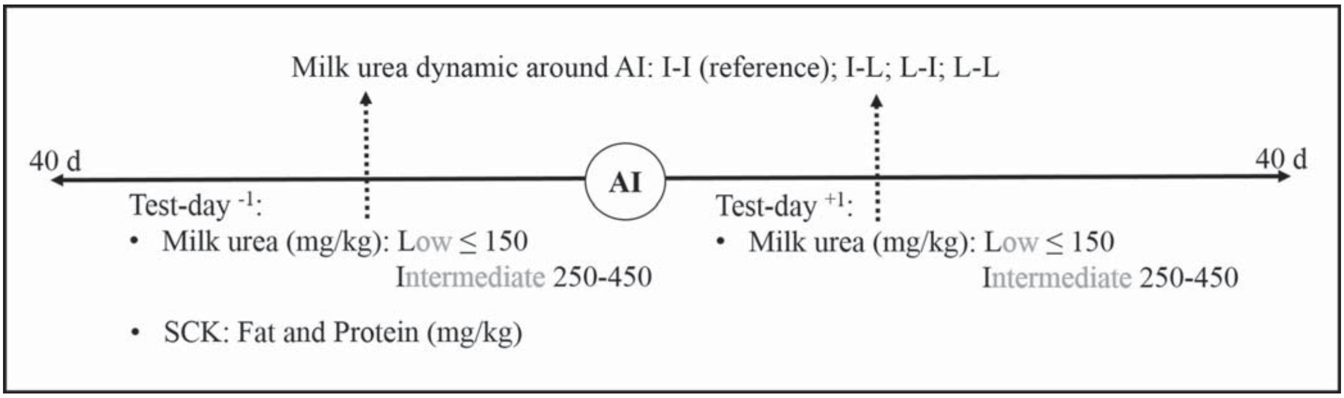

Figure 1. A schematic representation of the time of the AI and the measurements examined in this study. Milk urea was measured within 40 d before and after AI and transformed into a categorical variable representing the dynamic of its level around AI. A proxy of subclinical ketosis (SCK) was built using the fat and protein content (mg/kg) within 40 d before AI. I-I = intermediate milk urea levels (250–450 mg/kg) before and after AI; I-L = intermediate levels before and low levels (≤150 mg/kg) after AI; L-I = low levels before and intermediate levels after AI; L-L = low levels before and after AI.

Statistical Analysis

Data were analyzed using R (version 2.10.1, 2009–12–14, The R Foundation for Statistical Computing, Vienna, Austria). A 2-step statistical analysis was performed. The thresholds used to define the categorical variables included in the final logistic regression (milk urea, SCK, DIM, and 305-d MY) were first obtained through generalized additive models (package gam; https://cran.r-project.org/package=gam). Such models allowed us to determine the nature of the relationship between the response and the explanatory variables such that we were not required to assume a parametric relationship (Hastie and Tibshirani, 1990). Conception success for all AI was explained using continuous variables without any a priori assumptions. Herd was kept in all models as a random effect. This inclusion allowed us to identify the most efficient thresholds by which to define the classes of explanatory variables, such as milk urea, DIM at the time of AI, 305-d MY, and fat and protein contents. Separate univariate models were created with all of the above-mentioned explanatory variables, and the multivariate models were built by adding the significant variables ($P < 0.05$) to each model one by one.

The final logistic regression with a Poisson correction was then performed using the nlme package (https://cran.r-project.org/package=nlme). A log-link model

Table 1. Descriptive characteristics of the data set for the 4-yr study (2009–2012) and a statistical summary of some variables of interest for cows used in this work

Item	Year 2009	2010	2011	2012
Cows (no.)				
All breeds	706,593	736,906	738,516	649,683
Holstein	446,011	464,906	509,641	464,688
Mean DIM[1] ± SD (d)	143.1 ± 82.0	146.5 ± 86.8	149.2 ± 87.7	150.0 ± 87.9
Mean ± SD of 305-d MY[2] (kg)	8,312 ± 1,732	8,500 ± 1,712	8,756 ± 1,765	8,660 ± 1,789
All AI (no.)	1,129,843	1,191,627	1,201,047	1,038,501
Success rate (%)	35.9	36.2	34.9	37.2
First AI (no.)	541,522	568,148	569,989	498,109
Success rate (%)	39.6	40.2	38.7	41.5
Milk urea				
Mean ± SD (mg/kg)	247.4 ± 89.0	245.8 ± 91.9	261.2 ± 91.3	268.2 ± 86.6
Median (mg/kg)	240	250	259	265
1st percentile	60	60	70	85
99th percentile	480	490	502	494

[1]At the time of AI.

[2]Milk yield adjusted to a reference lactation period of 305 d.

with a Poisson distribution was used to calculate relative risks rather than odds ratios and to simultaneously address the lack of convergence sometimes observed in log binomial regressions (Greenland, 2004; Zou, 2004; Ospina et al., 2012). This final model included milk urea as a categorical variable and SCK as a binary variable, with a term of interaction between them, and was adjusted by lactation stage, milk yield, and parity. The logistic model included herd as a random variable and was applied yearly, first for Holstein cows only and then for cows of all breeds (Montbéliarde and Normande added).

RESULTS

The yearly mean milk urea concentrations ranged between 245.8 and 268.2 mg/kg (Table 1). The results of the generalized additive model showed high consistency between years for the shape of the relationship between conception success and milk urea concentrations before and after AI (Figure 2). A negative linear association between urea levels before AI and conception was observed. However, decreased conception success was observed in association with both low and high levels of milk urea after AI compared with average values. Values of 150 and 250 mg/kg (2.6 and 4.3 m*M*) were used as thresholds to distinguish low from intermediate urea levels. The generalized additive model also revealed the shape of the relationship between milk fat and protein content before AI and conception success (Supplemental Figure S1; https://doi.org/10.3168/jds.2016-12080). The explanatory variables enter the model as nonparametrically smoothed functions, and the obtained smooth curve resembled a step function. The smoothed lines suggest that AI are more successful when fat content ranges between 30 and 40 mg/kg, and then AI success decreases continuously and more sharply after a threshold of 45 mg/kg. In addition, protein contents between 28 and 35 mg/kg seem to be the most efficient for conception success.

First, the association between conception and urea was analyzed separately for urea levels before and after AI. The models were adjusted using the SCK proxy, DIM, 305-d MY, and parity, with herd as a random variable. The relative risks of conception were not significantly different for low urea (≤150 mg/kg) or intermediate levels (250–450 mg/kg) before AI (Table 2), but conception success was associated with low urea (≤150 mg/kg) after AI (Table 3); the decrease in conception in cows with low urea after AI was small (3 to 6%) but highly significant. A change in milk fat and protein (SCK proxy) was associated with a 16 to 20% decrease in conception rates. The interaction urea × SCK proxy was never significant and was excluded from the analyses. Artificial insemination was more successful when it was performed between 50 and 100 DIM compared with attempts outside this period. Within the Holstein breed, highly productive cows had an increased likelihood of conception compared with other cows, as did primiparous compared with multiparous cows (Tables 2 to 4). Across all breeds, highly productive cows were less likely to conceive (Supplemental Tables S1, S2, and S3; https://doi.org/10.3168/jds.2016-12080), but primiparous cows were still more likely to conceive. No differences in the results were observed when only first AI or all AI were included.

Second, conception success and change in urea levels between before and after AI were analyzed with adjustment by SCK proxy, DIM, 305-d MY, and parity, with herd as a random variable (Table 4). An increase in urea from values ≤150 mg/kg before AI to values of 250 to 450 mg/kg after AI (L-I group) was not associated with changes in conception success compared with the reference group. The opposite change; that is, a decrease in urea from 250 to 450 before AI to ≤150 mg/kg after AI (I-L group), was associated with a 5 to 9% decrease in conception success compared with cows with intermediate urea values (250–450 mg/kg) before and after AI (I-I group). No interaction was significant. Similar results were obtained when models were run for Holsteins only (Table 4) and for all breeds together (Supplemental Table S3; https://doi.org/10.3168/jds.2016-12080). In addition, cows of all breeds with low (≤150 mg/kg) milk urea levels before and after AI (L-L group) showed a 2 to 4% decrease in conception success compared with cows with urea values of 250 to 450 mg/kg before and after AI (I-I or reference group, Supplemental Table S3). This association was not significant when the model was applied to Holsteins only (Table 4). No differences in the results were observed when only the first AI or all AI attempts were included.

DISCUSSION

This work found a negative association between low urea and conception only for the period after AI, not for the period before AI (Table 2). Most previous studies analyzing the association between high urea levels and conception found a negative effect of high concentrations at or only after AI (Butler et al., 1996; Melendez et al., 2000), although some conflicting results have been reported. This result shows that the lack of effect of urea level before AI on conception success is also true for low levels of urea. Our results clearly show that low urea levels after AI are a risk factor for conception failure, as described for high levels after AI. This finding is in accordance with the trend toward reduced pregnancy rates for cows with milk urea concentrations <4.0 mM (233 mg/kg; Pehrson et al., 1992). The effect size reported in this work is limited, with an average 5% decrease in conception rates for low urea levels compared with intermediate urea levels after AI. This effect size is smaller than the previously reported average 5 to 40% decrease in conception associated with high urea levels after AI compared with moderate urea levels (in spite of large variations). Although small, the biological relevance of the 5% decrease in conception for low urea levels after AI remains important, considering

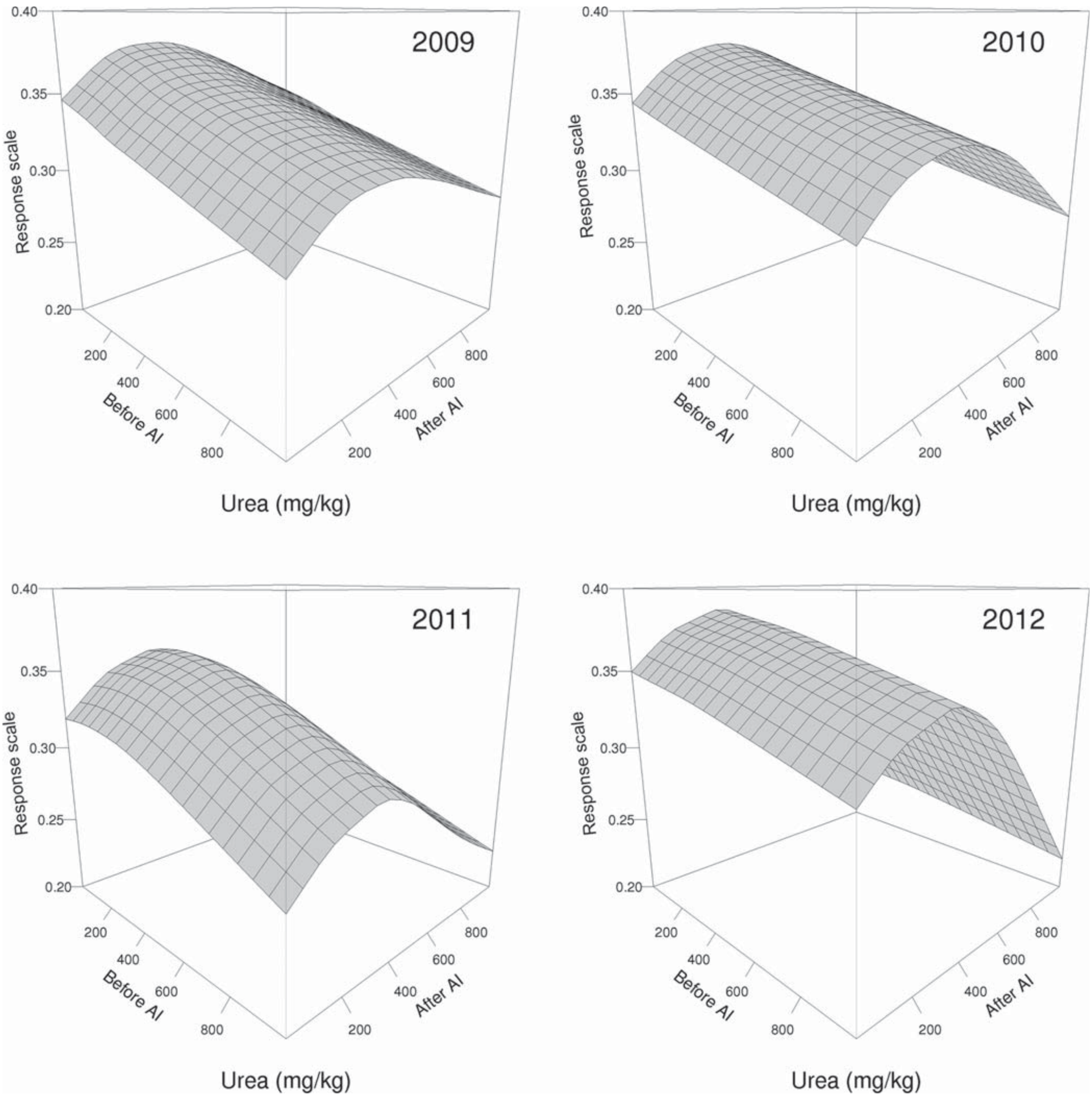

Figure 2. A 3-dimensional representation of the annual relationship (from 2009 to 2012) between milk urea levels before and after AI (as smooth functions) and relative values of conception success (response scale) at all AI in Holstein cows.

Table 2. Relative risk (95% CI) of conception at all AI associated with the explanatory variables for all years of the study when milk urea was evaluated before AI in Holstein cows

Item	Year			
	2009	2010	2011	2012
AI (no.)	148,513	175,950	177,935	155,302
Milk urea (mg/kg)				
250–450	Referent	Referent	Referent	Referent
≤150	1.01 (0.99–1.03)	1.02 (1.00–1.04)*	1.01 (0.99–1.03)	1.01 (0.99–1.04)
SCK[1]				
Without	Referent	Referent	Referent	Referent
With	0.84 (0.79–0.88)***	0.82 (0.77–0.86)***	0.83 (0.78–0.88)***	0.82 (0.77–0.87)***
DIM (d)				
50–150	Referent	Referent	Referent	Referent
<50	0.92 (0.83–1.00)†	0.79 (0.73–0.87)***	0.86 (0.78–0.95)**	0.89 (0.80–0.99)*
>150	0.88 (0.87–0.90)***	0.86 (0.84–0.88)***	0.86 (0.84–0.88)***	0.82 (0.81–0.84)***
305-d MY[2] (kg)				
<7,000	Referent	Referent	Referent	Referent
>7,000	1.04 (1.02–1.06)***	1.02 (1.00–1.04)*	1.06 (1.03–1.08)***	1.05 (1.03–1.08)***
Parity				
1st lactation	Referent	Referent	Referent	Referent
2nd lactation	0.91 (0.89–0.93)***	0.90 (0.89–0.92)***	0.91 (0.89–0.92)***	0.92 (0.91–0.94)***
3rd lactation	0.84 (0.82–0.87)***	0.85 (0.83–0.87)***	0.84 (0.82–0.86)***	0.84 (0.82–0.86)***
>3rd lactation	0.75 (0.73–0.77)***	0.74 (0.73–0.76)***	0.73 (0.72–0.75)***	0.74 (0.72–0.76)***

[1]Subclinical ketosis defined as fat content >45 mg/kg and protein content <28 mg/kg before AI.

[2]Milk yield adjusted to a reference period of 305 d.

***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; †$P < 0.1$.

the difficulties that farmers face in achieving sufficient reproductive performance and because conception success depends on many factors.

The main result of this work is that cows with intermediate urea levels (250–450 mg/kg) before AI and low urea levels (≤150 mg/kg) after AI (I-L group) had a 5 to 9% reduction in conception compared with cows in the I-I group and that no change in conception success was observed for cows that showed an increase urea levels from low (≤150 mg/kg) before AI to interme-

Table 3. Relative risk (95% CI) of conception at all AI associated with the explanatory variables for all years of the study when milk urea was evaluated after AI in Holstein cows

Item	Year			
	2009	2010	2011	2012
AI (no.)	150,589	176,730	179,256	153,984
Milk urea (mg/kg)				
250–450	Referent	Referent	Referent	Referent
≤150	0.97 (0.95–0.99)**	0.97 (0.95–0.99)**	0.96 (0.94–0.99)***	0.98 (0.95–1.00)†
SCK[1]				
Without	Referent	Referent	Referent	Referent
With	0.85 (0.81–0.90)***	0.81 (0.77–0.86)***	0.84 (0.79–0.89)***	0.83 (0.78–0.88)***
DIM (d)				
50–150	Referent	Referent	Referent	Referent
<50	0.87 (0.79–0.96)**	0.79 (0.72–0.86)***	0.83 (0.75–0.92)***	0.89 (0.80–0.99)*
>150	0.88 (0.86–0.90)***	0.86 (0.84–0.87)***	0.87 (0.85–0.88)***	0.83 (0.82–0.85)***
305-d MY[2] (kg)				
<7,000	Referent	Referent	Referent	Referent
>7,000	1.03 (1.01–1.05)*	1.01 (0.99–1.03)	1.06 (1.04–1.09)***	1.05 (1.03–1.07)***
Parity				
1st lactation	Referent	Referent	Referent	Referent
2nd lactation	0.92 (0.90–0.94)***	0.91 (0.89–0.93)***	0.91 (0.89–0.92)***	0.92 (0.90–0.94)***
3rd lactation	0.85 (0.83–0.87)***	0.85 (0.83–0.87)***	0.84 (0.82–0.86)***	0.84 (0.82–0.86)***
>3rd lactation	0.75 (0.73–0.77)***	0.74 (0.72–0.76)***	0.74 (0.72–0.75)***	0.74 (0.72–0.76)***

[1]Subclinical ketosis defined as fat content >45 mg/kg and protein content <28 mg/kg before AI.

[2]Milk yield adjusted to a reference period of 305 d.

***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; †$P < 0.1$.

diate after AI (250–450 mg/kg; L-I group) compared with cows in the I-I group. This result reveals that a decrease in urea levels around AI is deleterious for conception and suggests that farmers should consider this type of change in addition to the usual increase from intermediate to high urea levels near the time of AI. This result also confirmed that an increase in urea near the time of AI is only deleterious for conception at high to very high values (>450 mg/kg) of urea, whereas an increase from low to intermediate values is not a risk factor for conception failure.

A trend was found toward decreased conception in association with low urea levels before and after AI compared with intermediate urea levels before and after conception. This result addresses the increasing tendency toward very low permanent bulk milk urea observed in some herds and suggests that there is no evidence that low urea levels are more beneficial than intermediate urea levels. The negative effect on conception is limited and was only evident for L-L cows from all breeds and not for L-L Holstein cows, although this effect is unexplained.

The mechanisms by which urea concentration may affect reproduction are not clearly defined. They may involve effects on the hypothalamic-pituitary-ovarian axis through alterations to the LH pulse pattern and delays in the time of first ovulation after calving (Eldon et al., 1988) The effect may also be due to lower progesterone production and, consequently, lower conception rates at subsequent inseminations (Folman et al., 1973). In addition, increased urea concentration has been associated with a higher incidence of ovarian cysts (Ropstad and Refsdal, 1987). Other hypotheses suggest that this effect could be due to the potential toxicity of urea in the reproductive tract. Feeding high-CP diets might alter the uterine environment, including its ionic composition (Jordan et al., 1983), and decrease uterine pH (Elrod et al., 1993). Embryonic development was significantly decreased (18.2%) in the presence of 6 m*M* urea compared with the control group (23.9%) when it was evaluated at 7 and 9 d after start of maturation (De Wit et al., 2001). Oocyte cleavage rates were reduced for a high plasma urea N group compared with the control group, with subsequent effects on oocyte development to the blastocyst stage, fertilization, and blastocyst quality (Sinclair et al., 2000). In a more recent study, transferring embryos collected 7 d after insemination from the uteri of lactating

Table 4. Relative risk (95% CI) of conception at all AI associated with the explanatory variables for all years of the study when milk urea was evaluated through the dynamic of its levels before and after AI in Holstein cows

	Year			
Item	2009	2010	2011	2012
Milk urea category[1]				
I-I	Referent	Referent	Referent	Referent
(AI, no.)	75,106	84,746	97,865	94,647
I-L	0.95 (0.90–1.00)*	0.93 (0.90–0.98)**	0.91 (0.87–0.96)***	0.94 (0.89–1.00)*
(AI, no.)	4,989	6,598	5,644	3,699
L-I	0.97 (0.93–1.02)	1.01 (0.97–1.05)	0.99 (0.95–1.03)	1.00 (0.95–1.05)
(AI, no.)	5,634	6,599	6,558	4,097
L-L	0.98 (0.95–1.01)	1.00 (0.98–1.03)	0.99 (0.96–1.02)	0.99 (0.95–1.04)
(AI, no.)	13,066	16,948	10,640	6,136
SCK[2]				
Without	Referent	Referent	Referent	Referent
With	0.85 (0.79–0.90)***	0.80 (0.74–0.85)***	0.83 (0.77–0.88)***	0.81 (0.75–0.86)***
DIM (d)				
50–150	Referent	Referent	Referent	Referent
<50	0.89 (0.78–1.00)†	0.79 (0.70–0.88)***	0.85 (0.75–0.96)**	0.89 (0.78–1.01)†
>150	0.89 (0.87–0.92)***	0.86 (0.84–0.88)***	0.87 (0.85–0.89)***	0.82 (0.80–0.85)***
305-d MY[3] (kg)				
<7,000	Referent	Referent	Referent	Referent
>7,000	1.03 (1.00–1.06)*	1.02 (1.00–1.05)	1.07 (1.04–1.10)***	1.07 (1.04–1.10)***
Parity				
1st lactation	Referent	Referent	Referent	Referent
2nd lactation	0.92 (0.90–0.94)***	0.91 (0.89–0.93)***	0.91 (0.89–0.93)***	0.93 (0.90–0.95)***
3rd lactation	0.84 (0.82–0.87)***	0.85 (0.83–0.88)***	0.84 (0.82–0.87)***	0.84 (0.81–0.86)***
>3rd lactation	0.75 (0.73–0.78)***	0.75 (0.73–0.77)***	0.74 (0.72–0.76)***	0.75 (0.72–0.77)***

[1]I-I = intermediate milk urea levels (250–450 mg/kg) before and after AI; I-L = intermediate levels before and low levels (≤150 mg/kg) after AI; L-I = low levels before and intermediate levels after AI; L-L = low levels before and after AI.

[2]Subclinical ketosis proxy defined as fat content >45 mg/kg and protein content <28 mg/kg before AI.

[3]Milk yield adjusted to a reference period of 305 d.

***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; †$P < 0.1$.

cows with high plasma urea N concentrations resulted in lower pregnancy rates (11%) than did transferring embryos collected from cows with moderate plasma urea N levels (35%; Rhoads et al., 2006). Moreover, mechanisms by which low urea may affect reproduction remain unknown. Low milk urea may reflect inadequate dietary intake of other nutrients, and poor reproductive performance may result from a more severe energy deficit, a reduced N balance, or from mineral deficiencies (Westwood et al., 1998). In addition, the alteration in the uterus environment including uterine pH changes and alterations in the uterine secretory activity, as reported for high urea concentrations (Elrod and Butler, 1993), cannot be excluded.

Subclinical ketosis has been reported as a risk factor for deteriorated reproductive performance in dairy cows (Raboisson et al., 2014). In this study, a proxy of SCK was built using the fat and protein contents instead of the consensual definition based on BHB and nonesterified fatty acids. Due to the rapid mobilization of fat after parturition and the reduction in energy supply, SCK is characterized by an increase in milk fat and a decrease in milk protein. Thus, the thresholds of >45 and <28 mg/kg for milk fat and protein contents, respectively, were suggested as a proxy of SCK. The SCK proxy was associated with a 16 to 20% decrease in conception rate in this work, in accordance with a previously reported work that used a similar data set (Albaaj et al., 2016). Because of interactions between energy and protein metabolism at the cow level, an interaction between urea and SCK was expected in the current study. The interaction between energy and protein metabolism at early postpartum may arise, in part, from the energy cost for ammonia detoxification (Tamminga, 2006), which could be low or absent when the urea level is low and may partly explain the absence of such interaction in the present work. Further investigations that use shorter time periods and better SCK indicators are needed to more precisely define how energy and protein metabolism interact to affect conception and reproductive performance.

CONCLUSIONS

This work examined the association between milk urea concentrations and conception success with a focus on low urea levels. Urea levels ≤150 mg/kg (2.6 mM) were associated with decreased conception success. This association was observed only for urea level after AI, not for that before AI. A decrease in urea levels from intermediate before AI to low after AI was associated with reduced conception success, whereas an increase or a constant low level around AI seemed to be tolerable by cows. This work highlights the interest in monitoring urea as a tool to optimize reproductive performance. Previous works highlighted the negative effects of high urea concentrations on fertility. The present results suggest that low or instable urea levels may also negatively affect reproductive performance in cattle.

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Supplemental data

Table A1. Relative risk (95% CI) of conception at all artificial inseminations (AI) associated with the explanatory variables for all years of the study when milk urea was evaluated before AI and in cows of all breeds

Item	Year			
	2009	2010	2011	2012
AI (no.)	258,961	301,797	309,546	271,121
Milk urea (mg/kg)				
250-450	Reference	Reference	Reference	Reference
≤150	0.99(0.97-1.00)	0.99(0.97-1.00)†	0.99(0.97-1.00)†	1.00(0.98-1.02)
SCK[1]				
Without	Reference	Reference	Reference	Reference
With	0.80(0.77-0.84)***	0.79(0.76-0.83)***	0.82(0.78-0.85)***	0.81(0.77-0.84)***
DIM (days)				
50-150	Reference	Reference	Reference	Reference
<50	0.96(0.92-1.01)	0.91(0.88-0.95)***	0.96(0.92-1.01)	0.95(0.90-1.00)*
>150	0.83(0.82-0.84)***	0.83(0.82-0.84)***	0.83(0.82-0.84)***	0.81(0.80-0.82)***
305d MY[2] (kg)				
<7000	Reference	Reference	Reference	Reference
>7000	0.93(0.92-0.95)***	0.93(0.92-0.94)***	0.94(0.93-0.95)***	0.96(0.94-0.97)***
Parity				
1st lactation	Reference	Reference	Reference	Reference
2nd lactation	0.94(0.93-0.96)***	0.94(0.93-0.95)***	0.94(0.93-0.95)***	0.96(0.95-0.98)***
3rd lactation	0.89(0.87-0.90)***	0.89(0.88-0.91)***	0.89(0.87-0.90)***	0.90(0.88-0.92)***
>3rd lactation	0.80(0.79-0.82)***	0.79(0.78-0.81)***	0.79(0.77-0.80)***	0.80(0.78-0.81)***

[1]Subclinical ketosis defined as fat content > 45 mg/kg and protein content < 28 mg/kg before AI.

[2]Milk yield adjusted to a reference period of 305 d.

$^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; $^{\dagger}P < 0.1$.

Table A2. Relative risk (95% CI) of conception at all artificial inseminations (AI) associated with the explanatory variables for all years of the study when milk urea was evaluated after AI and in cows of all breeds

Item	Year			
	2009	2010	2011	2012
AI (no.)	262,386	304,392	311,173	269,352
Milk urea (mg/kg)				
250-450	Reference	Reference	Reference	Reference
≤150	0.95(0.93-0.96)***	0.94(0.93-0.96)***	0.95(0.93-0.96)***	0.97(0.95-0.99)**
SCK[1]				
Without	Reference	Reference	Reference	Reference
With	0.82(0.78-0.85)***	0.80(0.76-0.83)***	0.81(0.78-0.85)***	0.81(0.78-0.85)***
DIM (days)				
50-150	Reference	Reference	Reference	Reference
<50	0.95(0.91-0.99)*	0.92(0.88-0.95)***	0.95(0.91-0.99)*	0.96(0.92-1.01)
>150	0.83(0.82-0.84)***	0.83(0.82-0.84)***	0.83(0.82-0.84)***	0.82(0.80-0.83)***
305d MY[2] (kg)				
<7000	Reference	Reference	Reference	Reference
>7000	0.93(0.92-0.94)***	0.92(0.91-0.94)***	0.94(0.93-0.96)***	0.96(0.94-0.97)***
Parity				
1st lactation	Reference	Reference	Reference	Reference
2nd lactation	0.95(0.93-0.96)***	0.94(0.93-0.95)***	0.94(0.93-0.96)***	0.96(0.95-0.97)***
3rd lactation	0.89(0.88-0.91)***	0.89(0.88-0.91)***	0.88(0.87-0.90)***	0.91(0.89-0.92)***
>3rd lactation	0.81(0.79-0.82)***	0.79(0.78-0.81)***	0.79(0.77-0.80)***	0.80(0.79-0.82)***

[1]Subclinical ketosis defined as fat content > 45 mg/kg and protein content < 28 mg/kg before AI.

[2]Milk yield adjusted to a reference period of 305 d.

***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; †$P < 0.1$.

Table A3. Relative risk (95% CI) of conception at all artificial inseminations (AI) associated with the explanatory variables for all years of the study when milk urea was evaluated through the dynamic of its levels before and after AI and in cows of all breeds

Item	Year			
	2009	2010	2011	2012
Milk-urea category[1]				
II	Reference	Reference	Reference	Reference
(AI, no.)	135,414	154,711	175,603	166,094
IL	0.93(0.90-0.96)***	0.92(0.89-0.95)***	0.91(0.88-0.94)***	0.95(0.92-0.99)*
(AI, no.)	8,397	10,330	9,193	6,338
LI	0.97(0.94-1.00)†	0.99(0.96-1.02)	0.98(0.95-1.01)	0.99(0.96-1.03)
(AI, no.)	9,542	10,981	10,901	6,955
LL	0.97(0.94-0.99)**	0.96(0.94-0.98)***	0.97(0.94-0.99)*	0.98(0.95-1.02)
(AI, no.)	19,850	24,957	16,021	9,680
SCK[2]				
without	Reference	Reference	Reference	Reference
with	0.81(0.77-0.85)***	0.78(0.74-0.83)***	0.81(0.77-0.85)***	0.80(0.75-0.85)***
DIM (days)				
50-150	Reference	Reference	Reference	Reference
<50	0.97(0.91-1.02)	0.91(0.87-0.96)***	0.97(0.91-1.02)	0.96(0.91-1.02)
>150	0.84(0.82-0.85)***	0.83(0.81-0.84)***	0.84(0.82-0.85)***	0.81(0.80-0.82)***
305d MY[3] (kg)				
<7000	Reference	Reference	Reference	Reference
>7000	0.93(0.91-0.94)***	0.93(0.92-0.94)***	0.94(0.93-0.96)***	0.96(0.95-0.98)***
Parity				
1st lactation	Reference	Reference	Reference	Reference
2nd lactation	0.95(0.93-0.97)***	0.95(0.93-0.96)***	0.94(0.92-0.96)***	0.96(0.95-0.98)***
3rd lactation	0.89(0.87-0.91)***	0.90(0.88-0.92)***	0.88(0.87-0.90)***	0.90(0.88-0.92)***
>3rd lactation	0.81(0.79-0.83)***	0.80(0.79-0.82)***	0.79(0.77-0.80)***	0.80(0.78-0.82)***

[1]II: intermediate milk urea levels (250-450 mg/kg) before and after AI; IL: intermediate levels before and low levels (≤ 150 mg/kg) after AI; LI: low levels before and intermediate levels after AI; LL: low levels before and after AI.

[2]Subclinical ketosis proxy defined as fat content > 45 mg/kg and protein content < 28 mg/kg before AI.

[3]Milk yield adjusted to a reference period of 305 d.

***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; †$P < 0.1$.

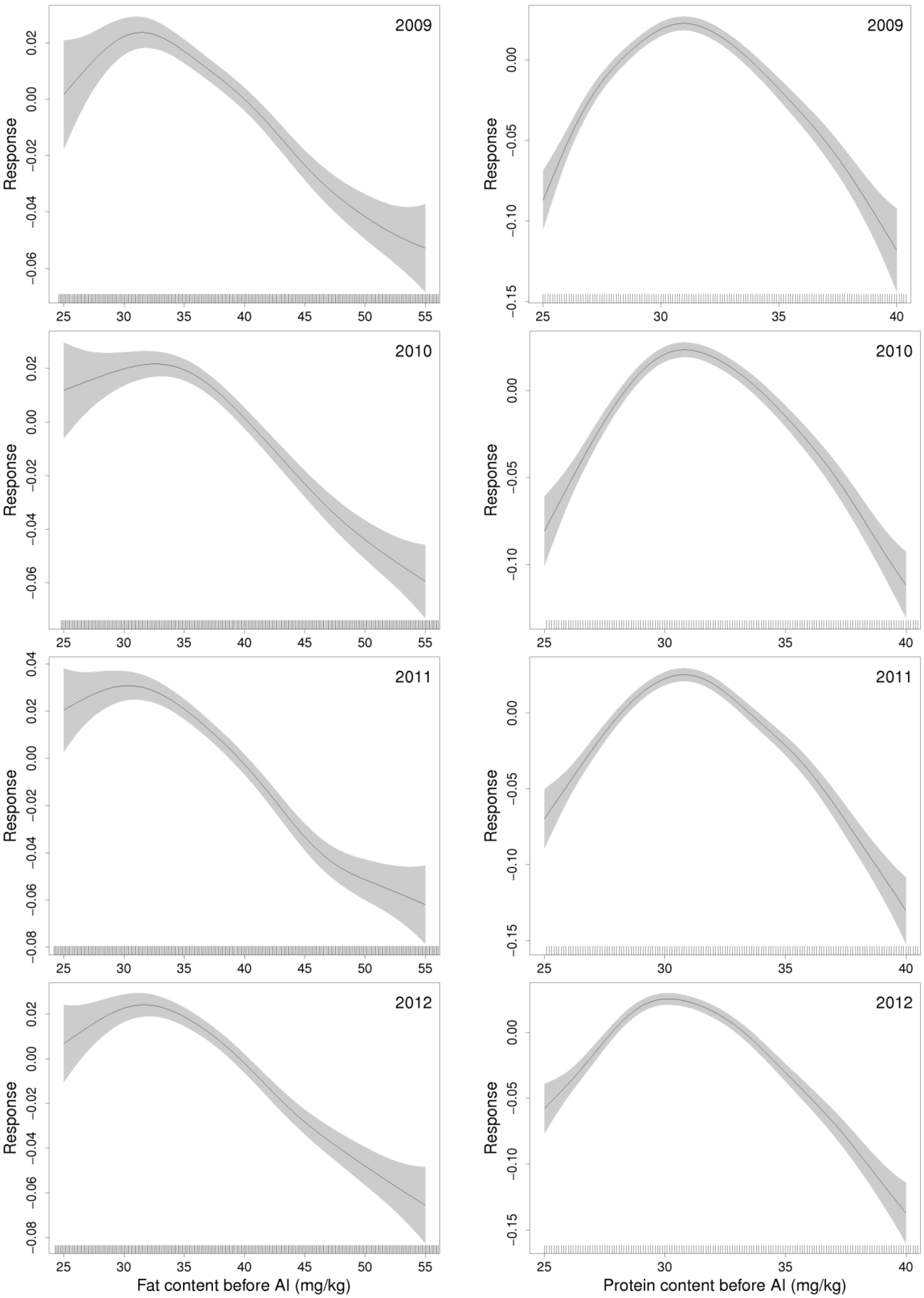

Figure A1. The association between fat (left) and protein (right) content (mg/kg) and the fitted response variable (conception success) centered on 0 for all years of the study.

5. Discussion générale

Améliorer la connaissance des facteurs de risque influençant la fonction de reproduction de la vache laitière, de leurs interactions, et de leurs poids relatifs, représente un enjeu crucial dans une logique d'intervention en élevage et d'orientation des systèmes productifs.

Les performances dégradées de la reproduction sont clairement associées, comme le montre la littérature abondante sur ce sujet, au niveau de production laitière, à la survenue d'une mammite clinique ou subclinique autour de l'IA, à la présence d'un déficit énergétique ou d'une hypercétonémie, et à la présence d'un excès de protéines dégradables dans la ration.

L'approche expérimentale proposée a d'abord permis d'identifier clairement l'interaction entre la cétose subclinique et les mammites dans leur impact sur la conception : la diminution de la conception en présence d'une mammite et d'une cétose subclinique est 2 fois plus grande que dans le cas d'une mammite isolée. Les associations conception - mammites et conception - cétose subclinique sont confirmées et évaluées de façon quantitative.

Une diminution de la concentration de l'urée dans le lait en dessous de 150 mg/kg (2,6 mmol/L) autour de l'IA a ensuite été associée à un risque de conception inférieur de 5 à 9% (Risque relatif [IC95%] = 0,91 [0,87-0,96] ; p < 0.001), par rapport à la situation où la concentration de l'urée est stable et comprise dans un intervalle de 250-450 mg/kg (4,3-7,7 mmol/L). Les variations à la baisse de la concentration de l'urée sont ainsi néfastes pour la conception, à l'instar des variations à la hausse autour de l'IA, même si ces dernières ont cependant un impact négatif bien plus fort.

5.1. Méthodologie retenue et interprétation

L'utilisation de modèles additifs généraux (GAM) permet de tracer la relation directe entre chaque prédicteur et les valeurs ajustées de la variable dépendante sans *a priori* (Hastie and Tibshirani, 1990). Le prédicteur dans ce modèle est additif et n'est pas obligatoirement linéaire. Pour cela, les variables prédictives dont la représentation est complexe sont introduites comme souples (smooth) et le GAM suppose que leur fonction puisse être approchée par une combinaison linéaire de fonctions non paramétriques. L'effet partiel de chacune des variables prédictives sur la variable à expliquer peut ainsi être mis en évidence de manière graphique, ce qui permet d'identifier les seuils les plus pertinents à utiliser pour créer des variables catégorielles.

En permettant d'introduire sans *a priori* les variables explicatives de la conception dans le modèle, la méthode permet de définir les classes de variables les plus pertinentes, dans le contexte donné de la variable expliquée.

Les différents résultats obtenus par les GAM restent en accord avec les distributions attendues : les seuils utilisés *in fine* sont globalement en accord avec ceux habituellement retenus ou décrits dans la littérature. Ceci est particulièrement vrai pour la définition de la cétose subclinique, et les seuils d'urée qui ont été retenus pour cette étude. La méthode et la représentation graphique retenue (Figure 2, publication n°1) a toutefois permis d'identifier la faible sensibilité de la conception aux CCS avant IA et au contraire la forte sensibilité de la conception aux CCS après IA.

Le modèle de régression de Poisson a été appliqué pour expliquer l'association entre la conception et plusieurs variables explicatives. Ce type de modèles permet : (i) d'éviter les problèmes de convergence qui sont souvent rencontrés lors des régressions binomiales ; et (ii) de calculer le RR directement via l'estimation du coefficient relatif à chaque variable explicative (Ospina et al., 2012). Il évite ainsi le calcul de l'OR, en pratique souvent interprété comme un RR. L'inconvénient de ce modèle vient du fait que l'intervalle de confiance est souvent surestimé lorsqu'il est appliqué à des variables binaires. L'utilisation de la régression de Poisson modifiée (avec variance robuste) a été proposée pour résoudre ce problème (Zou, 2004). Cette méthode a été testée sur nos données et les intervalles de confiance calculés ont été identiques pour les deux méthodes d'analyse (Annexe 2). Cette similitude des variances entre la régression de Poisson et la régression de Poisson modifiée provient de la taille importante du jeu de données mobilisé ici.

Les méthodes retenues ont inclus l'ensemble des données dans les modèles explicatifs. Elles n'ont pas évalué les qualités prédictives des modèles proposés à partir d'une sous-partie de la base de données, la totalité des données ayant servi à définir le modèle. L'objectif poursuivi ici reste majoritairement explicatif et ne repose pas sur la prédiction de la conception en fonction de certains paramètres. La prédiction de la conception est un objectif poursuivi par beaucoup d'auteurs et l'objet de nombreux travaux. La pertinence de cet objectif prédictif pourrait être discutée, en particulier dans des systèmes d'information peu fournis, ou seuls quelques paramètres de production ou de santé sont disponibles. Une étude récente a clairement conclu à la médiocrité de la prédiction de conception avec des modèles incluant comme variables la parité, le stade de lactation, la production laitière, la race, la saison, le rapport TB/TP et la note d'état corporel. Ces modèles estiment une probabilité de succès, qui ne peut être validée au niveau de la vache car le résultat de l'IA est binaire (gravide ou non) et n'est pas une probabilité (Rutten et al., 2016). Dans le cadre des travaux présentés ici, les données disponibles (et leur précision) rendent quasi caduques l'objectif de prédiction, compte tenu de leurs caractéristiques. C'est particulièrement le cas des définitions retenues pour la cétose subclinique, qui mériteraient d'être affinées. Une meilleure définition de la cétose subclinique, tel que proposé en perspectives, pourrait conduire à s'intéresser à la prédiction des modèles. Par ailleurs et de manière plus large, la revue de la littérature disponible a clairement identifié les limites liées à la prédiction de la conception. Par exemple, l'association entre les mammites cliniques et la conception a été clairement identifiée si la mammite intervient après l'IA (Lavon et al., 2011a). Ceci limite de fait la précision de la prédiction de la conception sur des critères, qui par définition, doivent être disponibles avant IA.

La validité de la base de données utilisée pour répondre aux objectifs fixés apparaît satisfaisante. En effet, riche de plusieurs millions de données, une multitude de sous-modèles a permis de tester différents temps où les évènements d'intérêt pouvaient survenir. Ainsi, les résultats de la publication n°1 ont été retrouvés de manière identique quel que soit la durée entre l'IA et le contrôle laitier avant et après l'IA. La principale limite liée aux caractéristiques des données provient d'une période relativement longue entre 2 contrôles successifs, ne permettant pas de dater précisément les évènements sanitaires (quand la hausse de CCS a-t-elle eu lieu par exemple). Cette base ne contient cependant pas les informations relatives aux avortements, donc non considérés dans la présente étude. Par ailleurs, l'usage de taureaux en élevage, fréquents lors d'infertilité ou après une série d'IA non fécondantes, n'a pas pu être considéré. L'usage simultané d'un taureau et d'une IA lors d'une même chaleur reste toutefois une pratique exceptionnelle.

Au final, les règles adoptées pour le nettoyage des données ont conduit à des taux de conception tels que ceux rapportés dans la littérature française, suggérant des méthodes adaptées. Le nombre d'IA exclues pour des raisons d'invraisemblance est resté faible (7%).

Au final, de nombreux indicateurs peuvent être retenues pour mesurer la fertilité et la fécondité. Ainsi, seul un indicateur partiel est mesuré ici. Bien qu'il soit l'un des plus pertinents, il reste limité compte tenu des multiples indicateurs de fertilité et fécondité envisageables.

5.2. Résultats

5.2.1. Taux de conception dans les systèmes français

De façon générale, il semble que la dégradation du taux de conception rapportée en France dans les années 1990 et au début des années 2000 s'est stabilisée durant la période de cette étude (2008-2012). Cette stabilité a été associée à une production laitière constante au cours de la même période, mais pourrait être expliquée par plusieurs autres facteurs, dont une plus grande sensibilité des éleveurs à ce problème. Les conditions du succès de la reproduction sont multifactorielles et l'arrêt de l'érosion de la réussite à l'IA peut avoir des origines multiples, dont une meilleure gestion de la reproduction (suivi plus fin des animaux, aide à la détection des chaleurs, recours à des traitements hormonaux, …), mais aussi des stratégies de gestion plus précises (IA ciblées…). Cette tendance à la stabilisation a déjà été décrite pour les vaches Prim'holstein françaises (Le Mézec et al., 2010a), ainsi qu'à l'étranger (LeBlanc, 2013).

Les différences de taux de conception entre les vaches Prim'holstein et les autres races laitières françaises peut provenir d'une aptitude raciale mais aussi de conditions d'élevages différentes selon la région et les systèmes d'élevage. Ce taux est plus élevé dans les régions où la proportion des vaches Montbéliarde est la plus grande (Massif Central, Rhône Alpes, Franche Comté et Savoie). Cependant, le taux de conception des seules vaches de race Prim'holstein reste plus élevé dans les bassins laitiers 7 et 8 (Massif central et Rhône Alpes), que dans les autres régions de la France. Ces variations régionales ont déjà été décrites en France (Barbat et al., 2010; Le Mézec et al., 2010a).

Des interactions saisonnières peuvent jouer un rôle dans cette variation régionale. En effets, les IA réalisées pendant la période froide de l'année ont plus de chance de succès que celles réalisées pendant les autres périodes (climat modéré ou chaud). L'effet saisonnier sur la conception est bien décrit, et peut être attribué entre autres, à l'effet négatif du stress thermique (Thatcher et al., 1974; Silva et al., 1992), aux différences alimentaires entre saisons, et à la distribution saisonnière des vêlages. La part des IA réalisées durant la saison froide en France est grande (> 50% entre octobre et janvier). De la même façon, les paramètres de fertilité sont meilleurs dans les régions où les vêlages sont regroupés en automne (est et nord-est de la France) que dans les régions où ils sont distribués sur toute l'année (Barbat et al., 2010). Pour une partie importante des vaches françaises, les rations évoluent fortement au cours de l'année. L'introduction de l'herbe représente par exemple entre autres à la fois un risque majeur d'excès de protéines dégradables, au moins pendant les quelques semaines suivant la mise à l'herbe, ou lors de la pâture des repousses automnales, mais aussi des risques de transition imparfaite et de déséquilibres alimentaires. Le stress thermique, en diminuant l'ingestion, favorise aussi les maladies métaboliques dont la cétose subclinique (Wheelock et al., 2010).

Les différents modèles proposés dans les 2 publications sont ajustés sur la parité, la production laitière et le stade de lactation (Jours en lait).

- Le taux de conception est réduit de façon linéaire quand la parité augmente. La hausse de la prévalence des affections de l'appareil reproducteur telles que les rétentions placentaires, les métrites et les kystes ovariens avec l'âge pourrait y contribuer fortement (Gröhn et al., 1989). Etonnamment, la probabilité de la conception augmente avec des niveaux de production laitière élevés en France. Cependant, les seuils à partir desquels cette augmentation est observée sont inférieurs chez les races Montbéliarde et Normande (5000 kg) que dans la race Prim'holstein (7000 kg). Les choix des seuils, relativement bas, peuvent expliquer pourquoi les vaches avec une production supérieure à ce seuil ont une meilleure conception que les autres. L'accroissement de la production laitière est souvent négativement associé à la fertilité. Cependant, cette relation semble complexe et fortement influencée par la parité, la gestion de l'élevage, la nutrition ou la génétique. Si l'accroissement de la production laitière s'est traduit par une réduction de la fertilité dans certaines études (Fonseca et al., 1983; Oltenacu et al., 1991), des conclusions opposées sont émises par d'autres (Eicker et al., 1996; Norman et al., 2009).
- Les chances de la conception augmentent quand elle est réalisée entre 50 et 100 jours de lactation. Cela est en accord avec la littérature où la fertilité augmente progressivement jusqu'au $60^{ème}$ jour post-partum, se maintient entre le $60^{ème}$ et le $120^{ème}$ jour puis diminue par la suite (Fulkerson, 1984; Hillers et al., 1984).

Par ailleurs, l'analyse des intervalles entre 2 IA successives montre un taux élevé de retours en chaleur tardifs (35 jours). Ceci témoigne d'une mortalité embryonnaire précoce fréquente, et/ou d'un défaut de détection du retour en chaleurs. De tels retours sont déjà constatés auparavant et peuvent contribuer à un allongement de l'intervalle vêlage-vêlage (Chevallier and Humblot, 1998).

5.2.2. Conception, mammites et déséquilibres alimentaires

La réduction de la conception lors de l'augmentation des CCS démontrée ici est en accord avec la littérature. En moyenne, la diminution associée à un niveau haut de CCS autour ou après l'IA est de 10%, 14%, 21% et 26% si le seuil de CCS utilisé est de respectivement 100 000 ; 200 000 ; 400 000 et 800 000 cellules/mL (résultats non rapportés). Quand le seuil de CCS est fixé à 150 000 cellules/mL (Fuenzalida et al., 2015), une augmentation durant une période de 2 jours en deça de 32 jours après l'IA a été associée à une baisse du taux de conception à l'IA1 de 12 points de pourcentage (44,6 vs. 36,5%, $P < 0,001$). Une augmentation des CCS au-delà de 200 000 et de 400 000 cellules/mL, comparé à des situations avec des CCS de < 20 000 cellules/mL, est associée à une baisse du taux de conception (risques relatifs [IC95%] de 0,84[0,79-0.89] et de 0,77[0,72-0,82]), respectivement (Hudson et al., 2012).

Les résultats proposés ici confortent clairement l'impact plus fort des mammites cliniques et subcliniques sur la conception lorsque la mammite a lieu après l'IA par rapport à une occurrence avant l'IA, tel que rapporté dans plusieurs publications (Hertl et al., 2010; Santos et al., 2004; Lavon et al., 2011a).

L'indicateur de la cétose subclinique retenu ici souffre d'un manque de sensibilité et de spécificité. Diagnostiquer la cétose subclinique à partir des valeurs de TB et de TP du lait conduit à des performances moyennes à médiocres, et particulièrement variables selon les seuils utilisés. Le recours aux taux pour évaluer les maladies métaboliques a certes été suggéré comme une alternative aux indicateurs biochimiques (Heuer et al., 2001). Les différences de prévalence de la cétose subclinique obtenues pour les différents critères diagnostiques utilisés (Tableau 2 de publication n°1), montrent bien les limites de la précision de cet indicateur, tels que précisé en synthèse (Table 6).
La biochimie sanguine représente sans ambiguïté le meilleur indicateur de la cétose subclinique, mais la nature des questions posées nécessite des bases d'informations de grandes dimensions et cette mesure n'est que rarement disponible. La mesure des corps cétoniques dans le lait via l'analyse des spectres moyen infrarouge (MIR) représente une alternative d'intérêt pour atteindre l'objectif poursuivi, malgré les limites de précision de cette méthode (Enjalbert et al., 2001).

5.2.3. Originalité des résultats proposés

Le lien complexe entre mammites, cétose subclinique et reproduction n'a jamais été décrit. Ce travail présente la première étude détaillée de cette interaction. La conception est d'autant plus réduite que les CCS sont plus élevées après l'IA et qu'une cétose subclinique est présente chez la vache. La baisse de conception en présence d'une mammite et d'une cétose subclinique par rapport à la situation où il y a seulement une mammite peut être jusqu'à 2 fois plus grande. L'association est d'autant plus grande que la cétose subclinique est présente avant l'IA pendant une période de 40 à 80 jours, montrant que l'effet négatif de la cétose subclinique sur la reproduction est décalé dans le temps. Dans une étude menée sur les vaches Prim'holstein, la proportion des vaches non gravides à 224 jours de lactation a été légèrement (P = 0,1) plus grand quand les vaches ont développé d'autres troubles en plus de la mammite clinique, comparé à la situation où la mammite clinique est la seule affection présente ou lorsque des troubles autres que la mammite sont présents (Ahmadzadeh et al., 2009).

D'un point de vue pratique, ces résultats montrent que la forte association mise en évidence entre certaines troubles conduit un animal malade à être plus sensible à d'autres troubles. Ces résultats suggèrent aussi qu'une inflammation locale peut affecter la réponse de l'ensemble de l'organisme et altérer les fonctions d'autres organes ou systèmes. De tels résultats n'ont jamais été décrits précédemment et conduisent à réévaluer l'impact négatif de ces affections sur la fertilité de la vache laitière. Ceci reste toutefois en accord avec une étude récente montrant que les brebis de lignées génétiques sélectionnées pour des CCS élevées ont une sensibilité au déficit énergétique plus élevée, avec des acides gras non estérifiés (AGNE) et d'acide bêta-hydroxybutyrique (BHBA) plus élevés lors d'une restriction énergétique, que les brebis de lignées génétiques sélectionnée pour des CCS faibles et qui sont plus résistantes aux mammites, renforçant l'idée qu'il existe une interaction entre les déséquilibres métaboliques et la sensibilité aux infections (Bouvier-Muller et al., 2016). Pour les raisons de séquences d'évènement évoquées précédemment, ces résultats n'apportent pas d'intérêt pratique dans un meilleur ciblage des vaches à inséminer. De nouveaux algorithmes intégrant la probabilité de mammites, en fonction de certains facteurs de risque et de l'état sanitaire de la mamelle, pourraient être utilisés à terme dans un indicateur synthétique indiquant le rapport coût/bénéfice de l'IA d'une vache donnée, dans la logique de l'élevage de précision.

Un déséquilibre azoté postpartum peut avoir des effets négatifs sur la fertilité des vaches laitières. Les valeurs de la concentration de l'urée sanguine et/ou du lait sont largement utilisées comme indicateurs de l'efficacité du métabolisme protéique. Des valeurs anormalement élevées ou au contraire faibles semblent être reliées à différents troubles de la fertilité. Les seuils de 7 mmol/L dans le sang ou de 420 mg/kg d'urée dans le lait ont été clairement identifiés comme associés à une diminution des chances de conception (Annexe 1). Ce travail a permis non seulement de bien quantifier le seuil supérieur d'urée à l'origine de la baisse de fertilité mais aussi de mettre en évidence une réduction très importante de la conception, qui peut aller jusqu'à 52%. La conception pourrait toutefois être réduite dès 6,5 mmol/L. Ces résultats suggèrent de ne pas considérer comme un facteur de risque de dégradation de la reproduction des troupeaux ou animaux avec des valeurs d'urée au-delà des recommandations traditionnelles d'urée du lait de 250-350 mg/kg mais plutôt de considérer un seuil de 420 mg/kg d'urée de lait ou 7.0 mmol/L d'urée dans le sang. Si certains auteurs ont trouvé un seuil similaire (6,9 mmol/L) d'urée sanguine et de lait avec un taux de gestation réduit ($P < 0{,}02$) (Butler et al., 1996a), les seuils rapportés par des autres sont largement différents, allant de 5,8 mmol/L (Chaveiro et al., 2011; Melendez et al., 2000) jusqu'à 9,1 mmol/L (Ferguson et al., 1993).

Tout comme pour les valeurs d'urée élevées, une baisse marquée de la concentration de l'urée du lait (< 150 mg/kg ; 2,6 mmol/L) autour de l'IA est un facteur de risque d'infertilité chez la vache laitière, bien que l'impact reste plus limité qu'en cas d'excès. Cette diminution de fertilité en cas d'urée faible autour de l'IA suggère de reconsidérer l'intérêt de cibler l'urée basse pour améliorer la fertilité et d'éviter la diminution de l'azote dans la ration lorsque les vaches sont mises à l'IA.

Les résultats mettent en évidence que la variabilité de l'urée sur des périodes courtes est aussi problématique pour des variations à la hausse (Annexe 1) qu'à la baisse (Publication 2). Des variations rapides de l'urée du sang ou du lait reflètent une instabilité du métabolisme azoté et ou énergétique chez les animaux, et est clairement associé associée à la performance de reproduction chez la vache laitière.

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6. Conclusions générales

Cette étude démontre clairement la présence d'une interaction entre l'inflammation mammaire et la cétose subclinique dans leur relation avec la réussite à l'IA. La présence d'une cétose subclinique module et amplifie le lien négatif entre mammite et fertilité. Ces résultats montrent ainsi que l'association mise en évidence entre certains troubles accroît la sensibilité de l'animal malade à d'autres troubles.

Les seuils de 7 mmol/L dans le sang ou de 420 mg/kg d'urée dans le lait ont été clairement identifiés comme associés à une diminution de moitié des chances de conception. Ces résultats suggèrent de ne pas considérer comme un facteur de risque de dégradation de la reproduction des troupeaux ou animaux avec des valeurs d'urée au-delà des recommandations traditionnelles d'urée du lait de 250-350 mg/kg.

Enfin, comme pour l'urée du lait ou du sang élevée, l'urée faible a été identifiée comme un facteur de risque d'infertilité chez la vache laitière, bien que son importance soit relative par rapport à des urées élevées. Pour des urées du lait ou du sang élevées ou basses, l'instabilité de l'urée autour de l'IA joue un rôle clé dans la baisse de la fertilité, les vaches ayant une capacité élevée d'adaptation à des valeurs anormales mais stables d'urée.

7. Perspectives

Si ce travail a permis de quantifier, pour la première fois, la force de l'interaction entre l'inflammation mammaire et la cétose subclinique dans la réduction de la réussite à l'IA, des travaux supplémentaires sont nécessaires afin de valider ces résultats originaux. D'une part, l'indicateur de cétose subclinique manque de sensibilité et de spécificité, et un indicateurs moins discutable est nécessaire pour une quantification claire de la force des interactions La validation des résultats présentés ici avec un indicateur plus précis de cétose subclinique, sur un échantillon de grande taille, est en cours. Les premiers résultats confirment la tendance décrite dans ce document.

En outre, les règles appliquées pour calculer le taux de réussite à l'IA dans cette étude sont relativement plus strictes que celles globalement définies dans la littérature. Certaines informations (telles que l'avortement, le recours à un taureau après un nombre fixé d'IA) ne sont pas fournies et le taux de réussite à l'IA peut être sous-estimé. Même si les taux calculés pour les principales races françaises sont cohérents avec ceux rapportés dans les autres études, il serait intéressant d'inclure plus d'informations pour améliorer la précision des résultats.

La déclinaison spatiale, par exemple pour les 10 bassins laitiers français, des modèles proposés pourraient par ailleurs permettre de nuancer certaines conclusions et de préciser les conditions de validé des résultats proposés. Les différents systèmes de production rencontrés dans chaque bassin laitier sont associés à des niveaux de production, des risques de maladies métaboliques et de déséquilibres azotés potentiellement assez différents, justifiant l'application des modèles nationaux à chaque bassin laitier indépendamment. De la même manière, les résultats ont montré de petites différences lorsque le modèle était appliqué à toutes les races française ou au contraire, limité aux vaches Prim'holstein. Différentes investigations pourraient permettre de mieux comprendre ces différences raciales.

8. Annexes

8.1. Annexe 1. Une synthèse des modes de calcul de plusieurs indicateurs de performance de la reproduction chez la vache laitière

Tableau 1. Récapitulatif des paramètres de fertilité et de fécondité souvent utilisé pour évaluer les performances de reproduction

Paramètre	Définition	Limites
Fertilité		
IA/IAF	$\frac{Nb\ total\ d'IA}{Nb\ d'IAF}$	
Taux de réussite en IA1	$\frac{NB\ d'IA\ suivies\ de\ gestation\ \text{à}\ 60\ jours\ (ou\ \text{à}\ 90\ jours)}{Nb\ d'IA1} \times 100$	
Taux de vêlage à l'IA1	$\frac{Nb\ de\ v\hat{e}lage\ suite\ \text{à}\ l'IA1}{Nb\ d'IA1} \times 100$	
Taux de vêlage à l'IA	$\frac{Nb\ de\ v\hat{e}lage\ suite\ \text{à}\ l'IA}{Nb\ d'IA} \times 100$	Biaisé par le nombre de vaches sorties de l'exploitation
Proportion des vaches > 2 IA	$\frac{Nb\ de\ vaches > 2\ IA}{Nb\ total\ d'IA1} \times 100$	Dépendent de la politique de réforme
Fécondité		
IV-IA1	Nombre de jours entre le vêlage et la première IA suivante	
%IV-IA1>90	$\frac{Nb\ de\ vaches\ \text{dont l'intervalle vêlage} - \text{IA1} > 90\text{j}}{Nb\ de\ vaches\ ins\acute{e}min\acute{e}es} \times 100$	
IV-IAF	Nombre de jours entre le vêlage et l'IA fécondante suivante	
%IV-IAF>110	$\frac{Nb\ de\ vaches\ \text{dont l'intervalle vêlage} - \text{IAF} > 110\text{j}}{Nb\ de\ vaches\ if\acute{e}cond\acute{e}es} \times 100$	
IV-V	Nombre de jours entre le vêlage et le vêlage suivant	- Connu tardivement - Ne prend pas en compte les réformes
IV−C1	Intervalle entre le vêlage et les premières chaleurs	
%IV-C1>60	$\frac{Nb\ de\ vaches\ \text{dont l'intervalle vêlage} - \text{premier chaleur} > 60}{Nb\ de\ vaches\ ins\acute{e}min\acute{e}es} \times 100$	Absence d'enregistrement systématique des premières chaleurs

8.2. Annexes 2. Urée élevée et fertilité chez la vache laitière : une méta-analyse

Résumé :

L'objectif de ce travail était de réaliser une méta-analyse sur la relation entre des niveaux élevés de l'urée sanguine ou du lait et la fertilité chez la vache laitière, afin de quantifier la force de cette relation et de proposer un seuil d'urée associé à la dégradation des résultats de fertilité.

L'ensemble des publications associant l'urée sanguine ou du lait (comme marqueur de l'excès de protéines dégradables) et les résultats de reproduction (principalement réussite à l'IA) a été revu et analysé statistiquement. Les 21 publications retenues regroupaient 61 modèles statistiques décrivant la conception en fonction d'un seuil de concentration de l'urée déterminé.

Les vaches avec des urémies ≥ à 7 mmol/L (420 mg/L) ont un risque de concevoir qui est 40 à 50% inférieur à celui des vaches avec des urémies plus basses (OR [CI95%] = 0,57 [0,45-0,73]). Le seuil de 6,5 mmol/L ne peut être formellement exclu. Par ailleurs, les animaux exposés aux rations riches en azote uniquement après l'IA ont plus de chance de concevoir que ceux exposés aux rations riches en azote avant l'IA, même si ce point reste à confirmer. En résumé, les excès de protéines dégradables de la ration à l'origine de la baisse de la conception peuvent être définis à partir de valeurs d'urée de 7 mmol/L (420 mg/L) et de manière encore plus certaine, au-dessus de 6,5 mmol/L (390 mg/L).

D'après cette méta-analyse, les concentrations en urée du tank inférieures à 390 voire 420 mg/L ne peuvent pas être retenues comme un facteur de risque de non réussite à l'IA, suggérant de revoir légèrement à la hausse la traditionnelle recommandation d'une concentration autour de 250-350 mg/L.

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High urea and pregnancy or conception in dairy cows: A meta-analysis to define the appropriate urea threshold

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ABSTRACT

Dietary proteins play an important role in reproduction, and increased dietary crude proteins, increased degradability of dietary proteins, and elevated blood or milk urea have been associated with decreased conception and pregnancy in many studies. The aim of this work was to provide a meta-analysis on the relationship between high milk or blood urea and pregnancy or conception, with a focus on defining the appropriate urea threshold associated with this issue. The meta-analysis included 61 different models from 21 papers. The thresholds of urea tested in the various models were built by steps of 1 mM urea. This constructed variable reduced heterogeneity by 61% in the meta-regression. The meta-analysis showed 43% lower odds of pregnancy or conception (odds ratio = 0.57; 95% confidence interval = 0.45–0.73) in cases where urea was ≥7.0 mM in the blood (plasma urea nitrogen = 19.3 mg/dL) or where urea was ≥420 mg/L in the milk compared with where urea values were lower. This threshold is the most suitable with regard to pregnancy or conception success, even if a threshold of 6.5 mM cannot be excluded with certainty. The results also highlighted the possibility of a stronger association between high urea concentrations and pregnancy or conception when high nitrogen exposure occurs before artificial insemination compared with after artificial insemination, but this possibility needs to be further studied. Whether the present results also apply to extensively pasture-based countries remains to be determined.
Key words: dairy cow, urea, nitrogen, reproduction

INTRODUCTION

Protein intake is an important determinant of dietary balance. Negative energy balance (**NEB**) is recognized as a significant challenge that dairy cows face during the early postpartum period. Diets that exceed the requirements for rumen degradation or contain nondegradable protein are often observed in the field (Butler, 1998). Urea is a good indicator of energy or protein imbalance and is a sensitive indicator of protein utilization efficiency (Kenny et al., 2002). Its small molecular size allows it to easily circulate in all fluids, and its concentration values are well correlated between milk and blood. Both milk and blood urea concentrations can be used to evaluate the nitrogen diet status of animals (Oltner and Wiktorsson, 1983; Baker et al., 1995). Dietary proteins play an important role in reproduction, and increased dietary CP, increased degradability of dietary proteins, and elevated blood or milk urea have been associated with decreased conception and pregnancy despite studies failing to highlight this association. Several previous studies (Westwood et al., 1998b; Leroy et al., 2008a,b) reviewed these relationships as well as the physiopathology behind the epidemiological link. In brief, diets high in dietary protein may interact with reproductive efficiency through (1) increased NEB linked to higher production and energy cost of desamination in the liver, (2) potential toxicity of the direct by-products of protein catabolism for the oocyte and the embryo, (3) prevention of the natural increase in uterine pH after ovulation and changes in the ionic composition of uterine fluid, (4) changes in $PGF_{2\alpha}$ secreted by the endometrial tissue, and (5) changes in the motility of spermatozoa (Butler, 1998; Westwood et al., 1998b; Melendez et al., 2003; Leroy et al., 2008a,b). The NEB promoted by high dietary protein also affects reproduction, including changes in the IGF-1, LH, and progesterone profiles (Leroy et al., 2008a). Interestingly, it seems that changes in dietary protein levels rather than high dietary protein levels alone may be involved in decreased reproductive performance. Lactating dairy cows can metabolically adapt to a prolonged high intake of quickly degradable protein, probably by opposing the adverse effects of long-term high concentrations of protein on embryo growth (Dawuda et al., 2002; Laven et al., 2004). There is still some uncertainty regarding whether the effect of high urea on reproduction occurs mostly during the period before, surrounding, or after AI (Hammon et al., 2000;

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Leroy et al., 2008b). The success of embryo transfer was decreased when the donors were fed high levels of dietary protein, whereas the result was not related to the urea nitrogen status of the recipients, suggesting a higher sensitivity of reproductive success to the factors preceding AI (Rhoads et al., 2006). The timing and duration of exposure need to be further studied, and their association with reproduction may depend on the parameter measured.

Many of the studies analyzing the association between dietary protein levels and reproduction used blood or milk urea (or urea nitrogen) as the gold standard to evaluate dietary nitrogen status. These studies were not specifically designed to address the association between urea concentration and pregnancy or conception, and the thresholds of urea used or defined in these studies were heterogeneous and arbitrary. Thus, the threshold of urea to be used in the field is ambiguous. Reviews on this topic have not focused on the threshold of urea or on the relative risk of decreased reproductive performance. This omission is concerning because these same studies are typically used to define the threshold to be achieved and to determine the relative risk of outcomes. This question has been intensively studied for subclinical ketosis (Raboisson et al., 2014). A quantitative review published in the 1990s included a small number of studies available on the topic. This work focused on conception (Westwood et al., 1998b). A review performed in 2003 gathered the available studies and highlighted the heterogeneity in the proposed thresholds of BUN or plasma urea nitrogen (**PUN**) for fertility but did not perform a formal meta-analysis (Melendez et al., 2003). Another study on reproduction performance did not evaluate urea but did evaluate dietary protein (Lean et al., 2012). The aim of this work was to provide a meta-analysis regarding the relationship between high milk or blood urea and reproductive performance, to propose the urea threshold for consideration in the field, and to clearly quantify the association between urea and pregnancy or conception.

MATERIALS AND METHODS

A literature search and screening process was conducted using the PubMed (http://www.ncbi.nlm.nih.gov/pubmed), CABI (www.cabi.org), and Google Scholar (http://scholar.google.com/) search engines to create a data set of papers using the key words "crude protein," "urea," "plasma urea nitrogen," "serum urea nitrogen," "reproduction," "conception," and "pregnancy" separately or in combination. New papers referenced by at least 1 of the papers identified in the previous step were also included.

Inclusion and Exclusion Criteria

To be included in the data set, the papers must have examined the odds ratios (**OR**) or equivalent of various reproductive performances, such as conception at service, pregnancy success, and so on, for cows or groups of cows with diets containing varying levels of urea or urea nitrogen. At this stage, no criteria regarding experimental design, including the number of animals per group, breed, urea measure (milk or blood), or use of hormones or synchronization protocols, were considered. Two types of studies were included: some studies reported the OR of reproduction performance change in the event of urea change (case 1), whereas others expressed the association differently but allowed us to obtain an expression for the OR through the construction of contingency tables (case 2). For this second situation, a temporary data set was implemented with the records of the mean urea values of groups of animals and the corresponding reproductive performances. Studies with in vitro experimental designs or with results without a clear estimation of the mean urea value for the different groups (with or without exposure to urea) were not included. Publications through June 2015 were included. All urea or nitrogen values were standardized (by dividing the urea nitrogen by 0.46 if BUN, PUN, or MUN) and are expressed in millimoles per liter. The models included at this stage refer to various outcomes: pregnancy after service (n = 28), conception after service (n = 18), conception after the first service (n = 15), number of AI attempts before successful AI (n = 8), interval from calving to first AI (n = 6), percentage of oocytes with division (n = 12), percentage of embryos that reach the blastocyst stage (n = 5), and viability of embryos (n = 7). Because raw data must be distributed as a 5-class categorical variable for the meta-regression, only outcomes with a minimal number of raw data (i.e., only the first 3 outcomes) were retained for the subsequent part of the study (Supplemental Tables S1 and S2; https://doi.org/10.3168/jds.2016-12009). They were collected and labeled "pregnancy or conception."

Twenty-one papers were selected. Most of the papers studied several outcomes or included more than 1 urea threshold, and 61 different models were recorded directly from the literature (case 1, n = 23) or through the implementation of a contingency table (case 2, n = 38). The final data set included the numbers of cows and herds studied, the energy density of the diet, the CP of the diet, the average milk production, the period (before, after, or both) of exposure to a high nitrogen level with respect to AI, the duration of high nitrogen level exposure, the date of sampling with respect to AI, the source used (blood or milk), whether pregnancy or conception was determined, the use of hormones or a synchronization protocol, the statistical method used (logistic regression, Poisson regression, raw data with contingency table), the threshold of urea used to classify the animals, the value of the OR or the change in the outcome and its 95% confidence interval (**CI**), the standard error or standard deviation, and the covariates included within the given models. The raw data of case 2 were modified for presentation as case 1 (OR calculated using temporary contingency tables). The threshold of urea to be considered was defined as the average of the mean urea values of the 2 groups of animals, and a variable gap was defined as the difference between the high or low urea mean values of the 2 groups of animals. The variable continuous threshold was then classified as the variables THRES and THRESbis according to the definitions reported in Table 1. The **THRES** and **THRESbis** are the categorical variables built with blood and milk urea concentration.

Statistical Analysis: Meta-Analysis

A meta-analysis was conducted on the extracted outcomes using the Metafor package (Viechtbauer, 2010) of R (version 3.0.2; R Foundation for Statistical Computing, Vienna, Austria). A fixed-effects model and a random-effects model were first used to estimate the log-effect size and its 95% CI and statistical significance. This process was conducted separately for each class of THRES or THRESbis as a reference. The inconsistency of results among trials was quantified using both Cochran's Q test and the I^2 statistic (Higgins et al., 2003). A value greater than 50% indicated substantial heterogeneity. If evidence of heterogeneity was found, a meta-regression analysis was subsequently performed to explore the sources of heterogeneity using the log-individual effect size for each trial as the outcome and a fixed-effects model or mixed-effects model with the random moderator study. The meta-regression was conducted by screening for the moderators period of exposure, duration of high nitrogen level exposure, date of sampling, source (blood or milk), method used to determine pregnancy, use of hormones or synchronization protocol, and gap (Table 2). The τ^2 values of the models, with or without moderators, were compared to explain the decrease in heterogeneity that occurred when the moderator was included in the model. Here, τ^2 denoted the amount of residual heterogeneity among the true effects—that is, the variability among the true effects that was not accounted for by the moderators included in the model. Forest plots were used to display the estimated effect size with its 95% CI and the final meta-regression adjustments (in gray). A sensitivity analysis was performed using funnel plots and influential case diagnostics. It included the analyses of externally standardized residuals, DFFITS (difference in fit) values, Cook's distances, covariance ratios, estimates of τ^2 and test statistics for (residual) heterogeneity when each study was removed in turn, hat values, and weights for the studies examining the odds of each outcome in the case of high urea.

Table 1. Raw data of the categories of urea THRES and THRESbis[1]

Category	Urea: Blood (mM)	Urea: Milk (mg/L)	n	Mean odds ratio (SD)
THRES	≤4.9	≤294	16	1.25 (0.98)
	5–5.9	300–354	13	0.85 (0.51)
	6–6.9	360–414	15	0.85 (0.34)
	7–7.9	420–474	10	0.47 (0.30)
	≥8	≥480	7	0.32 (0.28)
THRESbis	≤4.4	≤264	15	1.28 (1.00)
	4.5–5.4	270–324	8	1.05 (0.54)
	5.5–6.4	330–384	10	0.71 (0.31)
	6.5–7.4	390–444	18	0.69 (0.39)
	≥7.5	≥450	10	0.35 (0.25)

[1]THRES and THRESbis are the categorical variables built with blood and milk urea concentrations, according to thresholds defined in the table.

RESULTS

The descriptive statistics showed OR above 1 for low-urea classes of THRES and THRESbis and mean OR below 1 for high-urea classes (Table 1). Descriptive statistics for other variables are reported in Table 2. The heterogeneity of the data set describing the association between pregnancy or conception and high urea (Supplemental Table S1; https://doi.org/10.3168/jds.2016-12009) was high (I^2 = 82%; 95% CI = 53–93; Q statistics: χ^2 = 111, degrees of freedom = 60, $P <$ 0.001). The moderators THRES or THRESbis reduced the heterogeneity by 61 and 41%, respectively (Table 3, models 1 and 4). Using blood urea of 6 to 6.99 mM

Table 2. Raw data of other variables

Item	Category	n	Mean odds ratio (SD)
Period of exposure	Before and after AI	34	0.65 (0.44)
	Before AI	7	0.70 (0.46)
	After AI	20	1.19 (0.89)
Date of sampling	Before and after AI	23	0.74 (0.56)
	Before AI	13	0.59 (0.40)
	After AI	25	1.04 (0.80)
Source	Blood	38	0.80 (0.69)
	Milk	23	0.88 (0.62)
Synchronization	AI of cows in heat	53	0.84 (0.69)
	Synchronization	8	0.79 (0.48)
Pregnancy or conception	Pregnancy	28	0.62 (0.49)
	Conception	18	1.16 (0.80)
	Conception at first AI	15	0.83 (0.64)

as a reference showed 43% lower odds of pregnancy or conception (OR = 0.57; 95% CI = 0.45–0.73) when the urea was above 7.0 m*M*. The decrease in odds of pregnancy or conception was even greater if the urea was above 8.0 m*M* (mixed-effects model 1). Using urea of 6.5 to 7.49 m*M* as a reference showed 33% lower odds of pregnancy or conception when the urea was above 7.5 m*M* (mixed-effects model 4) and a significant increase in odds of pregnancy or conception for urea of 5.5 to 6.49 m*M* (fixed-effects model 4). Using urea ≤4.49 or 5 to 5.99 m*M* instead of 6 to 6.99 m*M* as a reference showed a significant decrease in the odds of pregnancy or conception for urea ≥7 m*M* (Supplemental Table S3; https://doi.org/10.3168/jds.2016-12009). Using urea of 7 to 7.99 m*M* as a reference showed a significant decrease in the odds of pregnancy or conception for urea ≥8 m*M* for the fixed-effects model. Similar results were observed for THRESbis (Supplemental Table S4; https://doi.org/10.3168/jds.2016-12009).

Including any other moderator in models 1 or 4 did not further decrease the heterogeneity of the data set and did not change the results (coefficients and *P*-values) observed for the moderator THRES or THRESbis. The moderators synchronization and source (milk or blood) were not significant for either the fixed-effects model or the mixed-effects model. In the fixed-effects model, exposure to high urea after AI was associated with improved odds of pregnancy or conception compared with exposure before and after AI (Table 3, model 2), and the OR of pregnancy or conception in cases of sampling before AI was 0.92 (95% CI = 0.86–0.99) compared with surrounding AI (Table 3, model 3). The inclusion of the moderator pregnancy or conception in models 1 or 4 showed an OR for success of AI of 0.81 (95% CI = 0.73–0.90) compared with conception at AI for the fixed-effects model. This finding led us to run models 1 and 4 on the 3 subpopulations of the moderator pregnancy or conception (Table 2). These 3 submodels clearly showed coefficients of interest similar to models 1 to 4 except for the nonsignificant association between THRES or THRESbis and the class conception. Models 1 to 4 performed using subpopulations of raw data cases (i.e., case 1 or 2 separately) showed results similar to those reported in Table 3.

The sensitivity analysis showed no evidence of publication bias in the funnel plot (Figure 1) and no outlier for the meta-regressions, but doubt exists for 3 to 5 raw data points (Supplemental Figure S1). Excluding these

Table 3. Results of meta-regressions

Model	Variable[1]	Category	Odds ratio of pregnancy or conception (95% CI): Fixed-effects model	Mixed-effects model
1	Intercept		0.96 (0.92–1.01)	0.81 (0.68–0.98)
	THRES	≤4.9 m*M* (≤294 mg/L)	0.93 (0.87–1.00)	0.95 (0.89–1.02)
		5–5.9 m*M* (300–354 mg/L)	0.95 (0.89–1.01)	0.96 (0.90–1.02)
		6–6.9 m*M* (360–414 mg/L)	Referent	Referent
		7–7.9 m*M* (420–474 mg/L)	0.47 (0.39–0.56)	0.57 (0.45–0.73)
		≥8 m*M* (≥480 mg/L)	0.26 (0.18–0.37)	0.45 (0.27–0.73)
2	Intercept		0.88 (0.82–0.96)	0.75 (0.57–1.00)
	THRES	≤4.9 m*M* (≤294 mg/L)	0.94 (0.89–1.00)	0.95 (0.89–1.02)
		5–5.9 m*M* (300–354 mg/L)	0.96 (0.90–1.01)	0.96 (0.91–1.03)
		6–6.9 m*M* (360–414 mg/L)	Referent	Referent
		7–7.9 m*M* (420–474 mg/L)	0.50 (0.41–0.61)	0.59 (0.46–0.74)
		≥8 m*M* (≥480 mg/L)	0.28 (0.20–0.40)	0.49 (0.29–0.82)
	Period of exposure	Before and after AI	Referent	Referent
		Before AI	0.96 (0.77–1.21)	0.97 (0.56–1.65)
		After AI	1.09 (1.02–1.17)	1.25 (0.81–1.92)
3	Intercept		0.96 (0.92–1.01)	0.80 (0.58–1.10)
	THRES	≤4.9 m*M* (≤294 mg/L)	0.95 (0.89–1.01)	0.95 (0.89–1.02)
		5–5.9 m*M* (300–354 mg/L)	0.96 (0.90–1.02)	0.97 (0.91–1.03)
		6–6.9 m*M* (360–414 mg/L)	Referent	Referent
		7–7.9 m*M* (420–474 mg/L)	0.48 (0.40–0.58)	0.58 (0.46–0.74)
		≥8 m*M* (≥480 mg/L)	0.27 (0.19–0.38)	0.49 (0.29–0.82)
	Date of sampling	Before and after AI	Referent	Referent
		Before AI	0.92 (0.86–0.99)	0.89 (0.55–1.44)
		After AI	1.10 (0.95–1.27)	1.21 (0.77–1.91)
4	Intercept		0.81 (0.73–0.91)	0.70 (0.55–0.89)
	THRESbis	≤4.4 m*M* (≤264 mg/L)	1.11 (0.99–1.25)	1.05 (0.89–1.24)
		4.5–5.4 m*M* (270–324 mg/L)	1.12 (1.00–1.26)	1.05 (0.89–1.23)
		5.5–6.4 m*M* (330–384 mg/L)	1.15 (1.02–1.29)	1.07 (0.91–1.26)
		6.5–7.4 m*M* (390–444 mg/L)	Referent	Referent
		≥7.5 m*M* (≥450 mg/L)	0.39 (0.29–0.52)	0.66 (0.44–0.99)

[1]THRES and THRESbis are the categorical variables built with blood and milk urea concentrations, according to thresholds defined in the table.

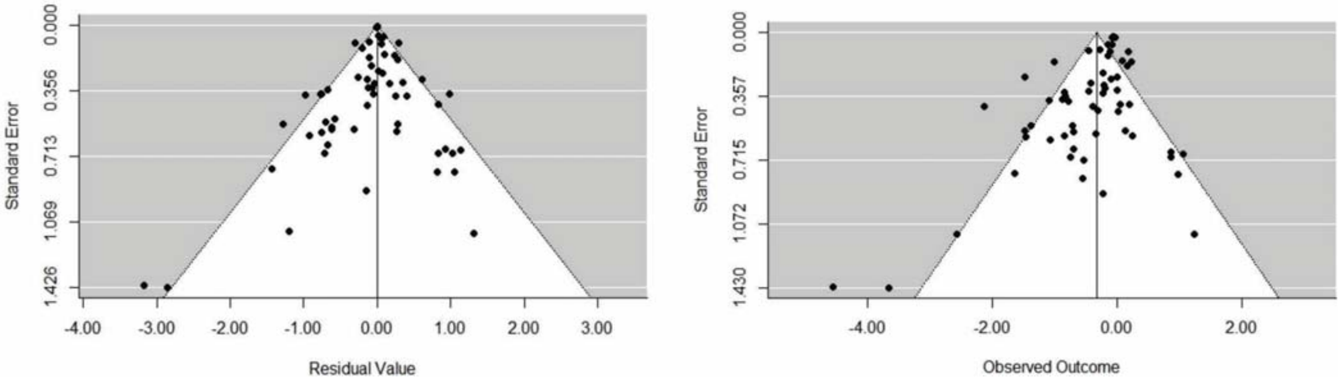

Figure 1. Funnel plots for the meta-regressions. Funnel plots of residual and observed risk of conception or pregnancy (displayed on the horizontal axis) for cows in cases of urea ≥7.0 m*M* in blood, including line of no effect and pseudo 95% confidence interval.

data did not lead to significant changes in either the significance of the results or the coefficients.

DISCUSSION

This work clearly demonstrates 43% lower odds of pregnancy or conception (OR = 0.57; 95% CI = 0.45–0.73) in cases of urea ≥7.0 m*M* in blood (PUN = 19.3 mg/dL) or urea ≥420 mg/L in milk compared with lower values. The OR can reach 0.55 in cases of very high urea (≥7.0 m*M* or 420 mg/L; BUN = 19.3 mg/dL) compared with moderate cases (7–8 m*M* or 420–470 mg/L; BUN = 19.3–22.1 mg/dL). The association between high urea and decreased pregnancy or conception appeared to be even stronger when urea above the threshold of 7 m*M* was considered high. The nonsignificant differences between classes of urea of 7 to 8 and >8 m*M* (Supplemental Table S3, model D; https://doi.org/10.3168/jds.2016-12009) are likely to be linked to the low statistical power because of the small number of cases in the highest urea class (n = 7). The tendency toward better pregnancy or conception in the reference class (urea = 6–7 m*M* or 360–420 mg/L) compared with lower classes, even if not significant (Tables 2 and 3), suggests a favorable moderate urea concentration and shows that no evidence supports pursuing a very low urea value to improve pregnancy or conception. This tendency was not observed for THRESbis (model 4), and only a few very low values of urea were included in the meta-analysis. The threshold 7.0 m*M* (model 1) is preferred to the threshold 6.5 m*M* (model 4) because the heterogeneity was lower with the inclusion of THRES compared with THRESbis. Lower odds of pregnancy or conception have already been observed in cases of urea ≥6.5 m*M* compared with lower values (model 4). The thresholds proposed here are (1) in accordance with those previously proposed in a review (6.9–7.2 m*M* of urea; BUN or MUN = 19–20 mg/dL; Butler, 1998) but (2) above the milk urea concentrations that are usually recommended in France (250–350 mg/L) or the objective (milk urea = 250–305 mg/L or MUN = 11.5–14.0 mg/dL) proposed in a recent review (Melendez et al., 2003). Such differences may arise from whether the recommended urea values were defined through (1) optimal and efficient use of dietary nitrogen in the rumen, with adjustment in dietary proteins or rumen fermentable energy needed in case of greater values (Melendez et al., 2003), or (2) significant odds of deterioration in reproduction performance observed in cases of urea above a certain threshold, as proposed here and elsewhere (Butler, 1998).

The second main conclusion is related to the association between pregnancy or conception and the period of high nitrogen exposure (fixed-effects model 2) or date of sampling (fixed-effects model 3). The study suggests a stronger association between high urea and pregnancy or conception when the exposure occurs before AI compared with after AI. This result is in accordance with the literature (Hammon et al., 2000; Rhoads et al., 2006) despite the inconsistent results. The mixed-effects models were not significant: the results of the 2 previous fixed-effects models (2 and 3) ultimately rely on the structure of the data set alone, suggesting extreme caution in the interpretation of this second conclusion. The data available for this study did not allow a rigorous evaluation of the association between the timing of protein interventions and the establishment of conception or maintenance of pregnancy. Dairy cows were recognized to be able to adapt to high urea concentration without any deterioration in reproduction performance provided that the urea concentration remains constant at high values and that there is a wash-out period before AI (Westwood et al., 1998b). This situation may be why high urea is recognized as a risk factor (with thresholds and odds ratios as indicated in this study) in most Northern countries, where livestock systems are most sensitive to urea change due to characteristics of feeding systems. In contrast, Australasian-type pasture-based systems with lower yielding cows may be less affected or unaffected by this issue (Westwood et al., 1998a,b; Laven et al., 2007; Ordonez et al., 2007).

This study was performed as recommended (Viechtbauer, 2010; Kovalchik, 2013). The final meta-regression (and relative adjusted OR) retained was judged on the reduction of the heterogeneity relative to regression without the moderator. The reduced heterogeneity allowed by the moderator THRES or THRESbis was expected because most of the raw data were obtained with various mean values or thresholds of urea. Models 1 and 4 should be considered the reference models of this study. As recommended by Kovalchik (2013), both fixed-effects and mixed-effects models were reported in this work. The fixed-effects meta-regression is a description of the k studies, and the random or mixed-effects meta-regression treats the k studies as a sample of a larger universe of studies. Because raw data may arise from the same study, the mixed-effects model is more robust in this situation than the fixed-effects model. The sensitivity analysis and the investigation of outliers showed the robustness of the results.

The assumptions in the way the data set was implemented have been validated through the results of the models. First, the models included in the data set referred to 3 closely related outcomes (OR of pregnancy, conception, or conception at first AI). Neither the behavior of this moderator (pregnancy, conception, or conception at first AI) in the main model (significant only in the fixed-effects model) nor the behavior of the 3 submodels performed separately for pregnancy, conception, or conception at first AI (with results similar to models 1 to 4) suggested considering these 3 topics separately. Second, neither the submodel performed with the data from case 1 alone nor the inclusion of the moderator gap in models 1 or 4 suggested any bias linked to the building of the data set with the 2 main types of data. Merging the 2 data sets made it possible to obtain enough data in each of the classes for moderator THRES or THRESbis. Third, the nonsignificant moderator source is consistent with the good correlation observed between milk and blood urea and validated the experimental design of this work, which did not distinguish the milk or blood urea. This result is consistent with the literature (Butler et al., 1996). Last, the CP and ME of the diets were not always reported in the studies, preventing their inclusion in the meta-regressions. This information might have helped highlight the complex relationship between energy and nitrogen supply.

CONCLUSIONS

This meta-analysis showed 43% lower odds of conception or pregnancy (OR = 0.57; 95% CI = 0.45–0.73) in cases where urea was $\geq$7.0 mM in blood (PUN = 19.3 mg/dL) or urea was $\geq$420 mg/L in milk compared with where urea values were lower. The results showed that this threshold is the most suitable with regard to pregnancy or conception, even if a threshold of 6.5 mM cannot be excluded with certainty. The results also highlighted the possibility of a stronger association between high urea and pregnancy or conception when the exposure occurs before AI compared with after AI, but this association needs to be confirmed through further studies. The results of this study reflect the data used in the study, which were obtained mostly from intensive production systems in the Northern Hemisphere and may not be applicable to Australasian-type pasture-based systems.

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Supplemental data

Table S1. Models included in the meta-analysis

Urea					Nitrogen exposure								
Threshold[1]	Mean[2]	Gap[3]	THRES[4]	THRESbis[5]	Period[6]	Duration (days)	Date of sampling[7]	Source (blood or milk)[8]	Synchronization[9]	Pregnancy or conception[10]	OR	Number of cows	Reference
	5.4	4.5	5	5	3	100	3		1	2	2.33	32	(Barton et al., 1996)
	6.9	1.9	6	7	31	86	3	1	2	1	1.07	629	(Burke et al., 1997)
	6.9	1.9	6	7	31	86	3	1	2	2	1.24	585	(Burke et al., 1997)
5.8			5	6	1	Na[11]	1	1	1	1	0.79	160	(Butler et al., 1996)
6.9			6	7	1	Na	1	1	1	1	0.79	160	(Butler et al., 1996)
7.9			7	8	1	Na	1	1	1	1	0.43	160	(Butler et al., 1996)
5.8			5	6	1	Na	1	1	1	1	0.34	155	(Butler et al., 1996)
6.9			6	7	1	Na	1	1	1	1	0.41	155	(Butler et al., 1996)
7.9			7	8	1	Na	1	1	1	1	0.63	155	(Butler et al., 1996)
9.1			8	8	1	Na	1	1	1	1	0.67	155	(Butler et al., 1996)
	6	2.6	6	6	3	89	3	1	1	3	0.48	63	(Canfield et al., 1990)
	6.7	1.9	6	7	3	240	3	1	1	1	0.71	59	(Chapa et al., 2001)
7.6	6.7	1.9	6	7	3	240	3	1	1	3	0.50	59	(Chapa et al., 2001)
7.7	6.9	1.7	6	7	31	240	3	1	1	1	0.47	63	(Chapa et al., 2001)
7.7	6.9	1.7	6	7	31	240	3	1	1	3	1.27	63	(Chapa et al., 2001)
5.8			5	6	1	Na	1		1	1	0.43	177	(Chaveiro. A. et al., 2011)
6.8			6	7	1	Na	1		1	1	0.33	177	(Chaveiro. A. et al., 2011)
7.9			7	8	1	Na	1		1	1	0.25	177	(Chaveiro. A. et al., 2011)
9			8	8	1	Na	1		1	1	0.08	177	(Chaveiro. A. et al., 2011)
6.7			6	7	1	Na	3	2	1	3	1.17	480	(Cottrill et al., 2002)
3.6			4	4	2	28	3	1	2	3	0.19	80	(Elrod and Butler, 1993)
5.8			5	6	2	28	3	1	2	3	0.23	80	(Elrod and Butler, 1993)
3.6			4	4	1	Na	3	1	1	1	0.73	332	(Ferguson et al., 1993)
5.4			5	5	1	Na	3	1	1	1	1.00	332	(Ferguson et al., 1993)
7.2			7	7	1	Na	3	1	1	1	1.02	332	(Ferguson et al., 1993)
9.1			8	8	1	Na	3	1	1	1	0.57	332	(Ferguson et al., 1993)
3.6			4	4	1	Na	3	1	1	2	0.65	332	(Ferguson et al., 1993)
5.4			5	5	1	Na	3	1	1	2	0.79	332	(Ferguson et al., 1993)
7.2			7	7	1	Na	3	1	1	2	0.82	332	(Ferguson et al., 1993)
9.1			8	8	1	Na	3	1	1	2	0.58	332	(Ferguson et al., 1993)
	3.1	0.2	4	4	3	122	3	1	1	2	0.43	60	(Folman et al., 1981)
	4.3	2.6	4	4	3	122	3	1	1	2	0.49	60	(Folman et al., 1981)
	4.1	1.7	4	4	2	13	13	1	2	1	1.20	113	(Gath et al., 2012)
	5.2	3.9	5	5	2	13	13	1	2	1	1.05	113	(Gath et al., 2012)
3.5			4	4	1	Na	2	2	1	2	0.88	4,017	(Godden et al., 2001)
4.5			4	5	1	Na	2	2	1	2	0.86	4,017	(Godden et al., 2001)

5.5			5	6	1	Na	2	2	1	2	0.92	4,017	(Godden et al., 2001)
6.5			6	7	1	Na	2	2	1	2	1.19	4,017	(Godden et al., 2001)
4.3			4	4	3	Na	1	2	1	2	0.92	36,000	(Hojman et al., 2004)
5.1			5	5	3	Na	1	2	1	2	0.94	36,000	(Hojman et al., 2004)
6.1			6	6	3	Na	1	2	1	2	0.95	36,000	(Hojman et al., 2004)
5.8			5	6	2	Na	2	2	1	3	0.86	1,073	(Melendez et al., 2000)
4.4	3.2	2.4	4	4	1	Na	4	1	1	1	2.33	43	(Miettinen, 1991)
4			4	4	3	35	1	2	1	3	2.84	263	(Pehrson et al., 1992)
5			5	5	3	35	1	2	1	3	0.81	263	(Pehrson et al., 1992)
6			6	6	3	35	1	2	1	3	0.99	263	(Pehrson et al., 1992)
7			7	7	3	35	1	2	1	3	0.46	263	(Pehrson et al., 1992)
	7.2	3.2	7	7	2	Na	3	1	2	1	0.23	82	(Rhoads et al., 2006)
	6	6.3	6	6	2	Na	3	1	2	1	1.13	82	(Rhoads et al., 2006)
	2.2	0.7	4	4	3	<100	3	1	1	2	0.79	39	(Rusche et al., 1993)
	2.6	1.6	4	4	3	<100	3	1	1	2	2.62	39	(Rusche et al., 1993)
	3.3	3	4	4	3	<100	3	1	1	2	3.43	39	(Rusche et al., 1993)
	7.3	2.1	7	7	1	Na	1	2	1	1	0.46	536	(Trevaskis and Fulkerson, 1999)
	7	4.3	7	7	1	Na	2	1	1	1	0.05	338	(Tshuma et al., 2014)
	9.5	0.7	8	8	1	Na	2	1	1	1	0.02	338	(Tshuma et al., 2014)
	10.8	1.9	8	8	1	Na	2	1	1	1	0.12	338	(Tshuma et al., 2014)
	12.5	1.6	8	8	1	Na	2	1	1	1	0.23	338	(Tshuma et al., 2014)
2			4	4	1	Na	2	2	1	3	0.90	2,153	(Wittwer et al., 1999)
4			4	4	1	Na	2	2	1	3	0.76	2,153	(Wittwer et al., 1999)
5			5	5	1	Na	2	2	1	3	0.63	2,153	(Wittwer et al., 1999)
7.3			7	7	1	Na	2	2	1	3	0.36	2,113	(Wittwer et al., 1999)

[1] threshold defined in the study; [2] threshold defined according to the mean urea value of the 2 groups of animals; [3] gap between the mean urea of the 2 groups of animals; [4] 1= ≤ 4.9 m*M* [≤ 294 mg/L], 2=5-5.9 m*M* [300-354 mg/L], 3=6-6.9 m*M* [360-414 mg/L], 4=7-7.9 m*M* [420-474 mg/L], 5=≥ 8 m*M* [≥ 480 mg/L]; [5] 1=≤ 4.4 m*M* [≤ 264 mg/L mg/L], 2=4.5-5.4 m*M* [270-324 mg/L], 3=5.5-6.4 m*M* [330-384 mg/L], 4=6.5-7.4 m*M* [390-444 mg/L], 5=≥ 7.5 m*M* [≥450 mg/L]; [6] 1= Before and after AI, 2= Before AI, 3= After AI; [7] 1= Around AI, 2= Before AI, 3= After AI; [8] 1= Blood, 2= Milk; [9] 1= AI of cows in heat, 2= Synchronization; [10] 1= Pregnancy, 2= Conception, 3 = Conception at first AI; [10] Not available.

Table S2. Papers excluded from the meta-analysis

Reference	Reason of exclusion
(Armstrong et al., 2001)	The outcome was follicular dynamics and embryo production
(Aller et al., 2013)	Out of scope (correlation blood/follicular fluid)
(Blanchard et al., 1990)	Diet nitrogen levels not expressed into urea
(Beran et al., 2013)	The outcome was cervical mucus urea
(Bruckental et al., 1989)	Conception rate not indicated
(Bruckental et al., 2000)	Out of scope (close urea concentration between groups)
(Carlsson and Pehrson, 1993)	Results did not allow OR calculation
(Carroll et al., 1988)	Out of scope (plasma urea not available for all parities)
(Carroll et al., 1994)	Out of scope (focused on fish meal, no urea)
(Dawuda et al., 2002)	The outcome was quality of embryos produced
(Garcia-Bojalil et al., 1994)	The outcome was quality of embryos/oocytes produced
(Guo et al., 2004)	Results did not allow OR calculation (regression provided)
(Gustafsson and Carlsson, 1993)	Presentation of results not compatible with OR calculation
(Hammon et al., 2005)	Out of scope (correlation blood/follicular fluid)
(Iwata et al., 2006)	Results did not allow OR calculation (correlation provided)
(Jackson et al., 2011)	Results did not allow OR calculation
(Kenny et al., 2002)	Out of scope (correlation blood/oviducts)
(Konig et al., 2008)	Results did not allow OR calculation (genetic correlation provided)
(Larson et al., 1997)	Results did not allow OR calculation (regression provided)
(Laven et al., 2002)	The outcome was embryos growth
(Law et al., 2009)	Diet nitrogen levels not expressed into urea
(McCormick et al., 1999)	Out of scope (confusion between crude protein and rumen degradation)
(Moriel et al., 2012)	Results did not allow OR calculation (regression provided)
(Ocon and Hansen, 2003)	The outcome was quality of embryos produced
(Ordonez et al., 2007)	Diet nitrogen levels not expressed into urea
(Rajala-Schultz et al., 2001)	Results did not allow OR calculation (regression provided)
(Reksen et al., 2002)	The outcome was the luteal function
(Ropstad and Refsdal, 1987)	Results did not allow OR calculation
(Santos et al., 2009)	The outcome was quality of oocytes produced
(Sinclair et al., 2000b)	Reproduction performances not included
(Sinclair et al., 2000a)	The outcome was quality of oocytes produced
(Sklan and Tinsky, 1993)	Out of scope (focus on by pass fat)
(Thompson et al., 2012)	Results did not allow OR calculation (correlation provided)
(Tillard et al., 2007)	Out of scope
(Son et al., 1996)	Results did not allow OR calculation (urea not reported)
(Wathes et al., 2007)	Results did not allow OR calculation (regression provided)
(Westwood et al., 2000)	Out of scope (close urea concentration between groups)

Table S3. Descriptive data of models included in the meta-analysis

Population	Protocol[1]	Metabolizable energy (Mcal)	CP (%)	Reference
64 cows, Holstein, Jersey		isocal[1]	13/20	(Barton et al., 1996)
641 heifers, Holstein	Trial	1.71	18.1/19.8	(Burke et al., 1997)
155 cows, Holstein	Epid	1.62	17.5 to 19	(Butler et al., 1996)
65 cows, Holstein	Trial	1.46/1.48	16,5/19.2	(Canfield et al., 1990)
64 heifers, 58 cows, Holstein	Trial		16.2/16.6/22.8	(Chapa et al., 2001)
177 cows, Holstein	Epid			(Chaveiro. A. et al., 2011)
480 cows, Holstein	Epid			(Cottrill et al., 2002)
80 cows, Holstein	Trial		15.4/21.8	(Elrod and Butler, 1993)
332 cows, Holstein	Epid	isocal		(Ferguson et al., 1993)
60 cows, Holstein	Trial		15.9/16.6/20.2	(Folman et al., 1981)
60 cows, Beef	Trial			(Gath et al., 2012)
60 herds, Holstein	Epid			(Godden et al., 2001)
36,073 cows, Holstein	Epid	1.75	17.2	(Hojman et al., 2004)
1,073 cows, Holstein	Epid	1.76	18.6	(Melendez et al., 2000)
45 cows, Finland	Trial			(Miettinen, 1991)
386 cows	Trial			(Pehrson et al., 1992)
23 cows, 122 heifers, Holstein	Trial	0.4/1.7	9.6/15.7/21.9/24.4	(Rhoads et al., 2006)
40 cows, Angus Herford	Trial	1.47		(Rusche et al., 1993)
556 cows, Holstein	Epid			(Trevaskis and Fulkerson, 1999)
418 cows, Beef	Trial			(Tshuma et al., 2014)
2,754 cows, Holstein	Epid			(Wittwer et al., 1999)

[1] Epid=Epidemiological approach; [2] Isocaloric values in all groups

Table S4. Results of the meta-regression with various reference classes for the THRES variable

			OR of pregnancy [95%IC]	
			Fixed-effect model	Mixed-effect model
Model A	Intercept		0.90 [0.86 – 0.94]	0.77 [0.64 – 0.93]
	THRES	≤ 4.9 m*M* [≤ 294 mg/L]	Reference	Reference
		5-5.9 m*M* [300-354 mg/L]	1.01 [0.96 – 1.08]	1.01 [0.95 – 1.07]
		6-6.9 m*M* [360-414 mg/L]	1.07 [1.00 – 1.14]	1.05 [0.98 – 1.12]
		7-7.9 m*M* [420-474 mg/L]	0.5 [0.42 – 0.60]	0.60 [0.48 – 0.76]
		≥ 8 m*M* [≥ 480 mg/L]	0.28 [0.20 – 0.39]	0.47 [0.28 – 0.77]
Model B	Intercept		0.91 [0.88 – 0.95]	0.78 [0.65 – 0.94]
	THRES	≤ 4.9 m*M* [≤ 294 mg/L]	0.99 [0.93 – 1.04]	0.99 [0.93 – 1.04]
		5-5.9 m*M* [300-354 mg/L]	Reference	Reference
		6-6.9 m*M* [360-414 mg/L]	1.06 [0.99 – 1.12]	1.03 [0.98 – 1.10]
		7-7.9 m*M* [420-474 mg/L]	0.49 [0.41 – 0.59]	0.60 [0.47 – 0.75]
		≥ 8 m*M* [≥ 480 mg/L]	0.27 [0.19 – 0.39]	0.46 [0.28 – 0.76]
Model C	Intercept		0.45 [0.38 – 0.54]	0.47 [0.36 – 0.61]
	THRES	≤ 4.9 m*M* [≤ 294 mg/L]	2.00 [1.66 – 2.40]	1.66 [1.31 – 2.09]
		5-5.9 m*M* [300-354 mg/L]	2.03 [1.68 – 2.44]	1.68 [1.33 – 2.11]
		6-6.9 m*M* [360-414 mg/L]	2.14 [1.78 – 2.58]	1.74 [1.38 – 2.20]
		7-7.9 m*M* [420-474 mg/L]	Reference	Reference
		≥ 8 m*M* [≥ 480 mg/L]	0.55 [0.38 – 0.82]	0.78 [0.46 – 1.31]
Model D	Intercept		0.25 [0.18 – 0.35]	0.36 [0.22 – 0.59]
	THRES	≤ 4.9 m*M* [≤ 294 mg/L]	3.60 [2.55 – 5.08]	2.14 [1.30 – 3.51]
		5-5.9 m*M* [300-354 mg/L]	3.65 [2.59 – 5.15]	2.16 [1.31 – 3.55]
		6-6.9 m*M* [360-414 mg/L]	3.86 [2.73 – 5.44]	2.24 [1.36 – 3.69]
		7-7.9 m*M* [420-474 mg/L]	1.80 [1.22 – 2.65]	1.29 [0.77 – 2.17]
		≥ 8 m*M* [≥ 480 mg/L]	Reference	Reference

Table S5. Results of the meta-regression with various reference classes for the THRESbis variable

			OR of pregnancy [95%IC]	
			Fixed-effect model	Mixed-effect model
Model A	Intercept		0.91 [0.86 – 0.95]	0.74 [0.59 – 0.93]
	THRESbis	≤ 4.4 m*M* [≤ 264 mg/L mg/L]	Reference	Reference
		4.5-5.4 m*M* [270-324 mg/L]	1.01 [0.95 – 1.07]	1.00 [0.94 – 1.06]
		5.5-6.4 m*M* [330-384 mg/L]	1.04 [0.97 – 1.11]	1.02 [0.96 – 1.09]
		6.5-7.4 m*M* [390-444 mg/L]	0.90 [0.80 – 1.01]	0.95 [0.81 – 1.12]
		≥ 7.5 m*M* [≥450 mg/L]	0.35 [0.27 – 0.46]	0.63 [0.42 – 0.95]
Model B	Intercept		0.91 [0.89 – 0.95]	0.73 [0.58 – 0.92]
	THRESbis	≤ 4.4 m*M* [≤ 264 mg/L mg/L]	0.99 [0.93 – 1.05]	1.00 [0.94 – 1.06]
		4.5-5.4 m*M* [270-324 mg/L]	Reference	Reference
		5.5-6.4 m*M* [330-384 mg/L]	1.02 [0.97 – 1.09]	1.02 [0.96 – 1.09]
		6.5-7.4 m*M* [390-444 mg/L]	0.89 [0.79 – 1.00]	0.95 [0.81 – 1.12]
		≥ 7.5 m*M* [≥450 mg/L]	0.35 [0.27 – 0.46]	0.63 [0.43 – 0.95]
Model C	Intercept		0.94 [0.90 – 0.98]	0.75 [0.60 – 0.94]
	THRESbis	≤ 4.4 m*M* [≤ 264 mg/L mg/L]	0.96 [0.90 – 1.03]	0.98 [0.92 – 1.04]
		4.5-5.4 m*M* [270-324 mg/L]	0.97 [0.91 – 1.03]	0.98 [0.92 – 1.04]
		5.5-6.4 m*M* [330-384 mg/L]	Reference	Reference
		6.5-7.4 m*M* [390-444 mg/L]	0.87 [0.77 – 0.97]	0.93 [0.79 – 1.10]
		≥ 7.5 m*M* [≥450 mg/L]	0.34 [0.26 – 0.44]	0.92 [0.42 – 0.93]
Model D	Intercept		0.32 [0.24 – 0.42]	0.47 [0.31 – 0.71]
	THRESbis	≤ 4.4 m*M* [≤ 264 mg/L mg/L]	2.84 [2.17 – 3.71]	1.58 [1.05 – 2.36]
		4.5-5.4 m*M* [270-324 mg/L]	2.86 [2.19 – 3.74]	1.57 [1.05 – 2.35]
		5.5-6.4 m*M* [330-384 mg/L]	2.94 [2.25 – 3.85]	1.61 [1.07 – 2.40]
		6.5-7.4 m*M* [390-444 mg/L]	2.56 [1.92 – 3.40]	1.50 [1.00 – 2.24]
		≥ 7.5 m*M* [≥450 mg/L]	Reference	Reference

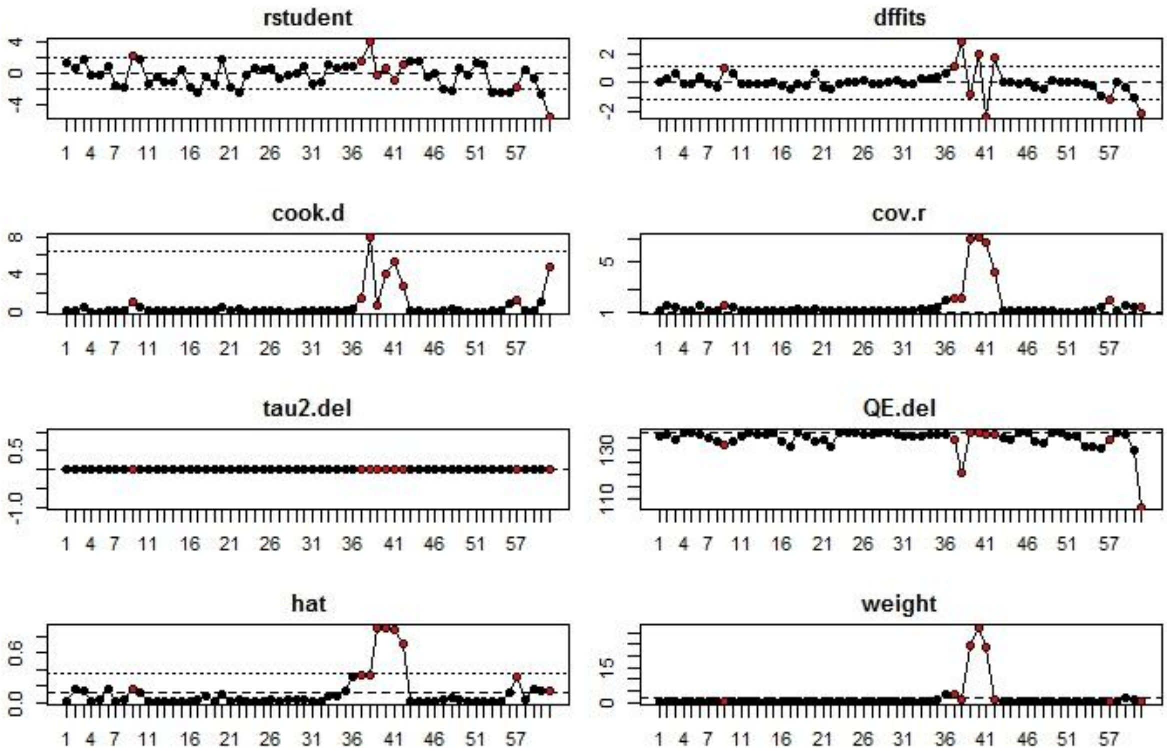

Figure S1. Influential graph of the meta-regression (model 2)

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8.3. Annexes 3. Comparaison des intervalles de confiance obtenus par la correction de Poisson et la correction de Poisson modifiée

En régression logistique, la correction de poisson est recommandée pour (i) permettre de calculer le risque relatif au lieu de l'odds-ratio (ii) éviter les problèmes de convergence souvent rencontrés en régression binomiale lors de l'utilisation des grosses bases des données. L'inconvénient de cette méthode est que l'intervalle de confiance est souvent surestimé pour les variables binaires. Une correction de poisson modifiée a été proposée afin de résoudre ce problème. Cependant, les intervalles de confiance calculés lors l'application de ces deux méthodes sur la base de données utilisée dans ce travail ont été très proches comme montré dans cet annexes. Cela peut être justifié par la taille importante de jeu de données utilisé.

Comparison of confidence intervals obtained via the Poisson regression and the modified Poisson regression:

The following examples provide the 95% confidence intervals for RR calculated in our models via the Poisson regression (before last column) and those calculated via the modified Poisson regression with a robust error variance (last column), which was proposed by (Zou, 2004) to deal with variance overestimation when Poisson regression is applied to binary data. They clearly show that the 95% confidence intervals were identical for both methods of analysis. This similarity of the variances between the Poisson regression and the modified Poisson regression is due to the large size of the data set used here.

Example 1:

Table 1. Relative risk of conception associated with the different explanatory variables and their confidence intervals calculated via the Poisson regression or the modified Poisson regression models; subpopulation retained by SCK2 (Number of obs: 362440)

	Estimate	SE	Corrected SE	RR	95%CI	95%CI (corrected)
Class of SCC						
LL	Referent					
LH	-0.131	0.01	0.008	0.88	(0.86-0.89)	(0.86-0.89)
HL	-0.028	0.01	0.008	0.97	(0.95-0.99)	(0.96-0.99)
HH	-0.145	0.008	0.006	0.86	(0.85-0.88)	(0.85-0.87)
SCK1						
Without	Referent					
With	-0.169	0.014	0.012	0.84	(0.82-0.87)	(0.82-0.86)
DIM (days)						
50-100	Referent					
< 50	-0.146	0.022	0.018	0.86	(0.83-0.90)	(0.83-0.89)
> 100	-0.093	0.006	0.005	0.91	(0.90-0.92)	(0.90-0.92)
305-day MY (kg)						
< 7000	Referent					
> 7000	0.021	0.006	0.005	1.02	(1.01-1.03)	(1.01-1.03)
Parity						
1st	Referent					
2^{nd}	-0.091	0.007	0.005	0.91	(0.90-0.93)	(0.90-0.92)
3rd	-0.148	0.008	0.006	0.86	(0.85-0.88)	(0.85-0.87)
> 3rd	-0.305	0.009	0.007	0.74	(0.72-0.75)	(0.73-0.75)

Example 2:

Table 2. Relative risk of conception associated with the different explanatory variables and their confidence intervals calculated via the Poisson regression or the modified Poisson regression models; subpopulation retained by SCK1 (Number of obs: 815933)

	Estimate	SE	Corrected SE	RR	95% IC	95% IC (corrected)
Class of SCC						
LL	Referent					
LH	-0.134	0.007	0.006	0.87	(0.86-0.89)	(0.86-0.89)
HL	-0.032	0.007	0.006	0.97	(0.96-0.98)	(0.96-0.98)
HH	-0.144	0.006	0.005	0.87	(0.86-0.88)	(0.86-0.87)
SCK1						
Without	Referent					
With	-0.056	0.004	0.003	0.95	(0.94-0.95)	(0.94-0.95)
DIM (days)						
50-100	Referent					
< 50	-0.15	0.014	0.012	0.86	(0.84-0.88)	(0.84-0.88)
> 100	-0.069	0.004	0.003	0.93	(0.93-0.94)	(0.93-0.94)
305-day MY (kg)						
< 7000	Referent					
> 7000	0.011	0.004	0.003	1.01	(1.0-1.02)	(1.01-1.02)
Parity						
1st	Referent					
2nd	-0.105	0.005	0.004	0.90	(0.89-0.91)	(0.89-0.91)
3rd	-0.185	0.006	0.004	0.83	(0.82-0.84)	(0.82-0.84)
> 3rd	-0.337	0.006	0.005	0.71	(0.71-0.72)	(0.71-0.72)